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COMPARATIVE EFFECT OF ACTH, CORTISONE AND DCA ON RENAL FUNCTION, ELECTROLYTE EXCRETION AND WATER EXCHANGE IN NORMAL DOGS

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THE inadequacies of our knowledge of the role of adrenocortical hormones in the regulation of electrolyte and water metabolism are becoming increasingly apparent (Gaunt, *et al.*, 1949; Ingle, 1950; Sayers, 1950). Extensive and numerous observations on the action of DCA have been reported (Ragan, *et al.*, 1940; Ferrebee, *et al.*, 1941; Mulinos, *et al.*, 1941; Zierler and Lilienthal, 1948; Gaudino and Levitt, 1949; Davis, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952), but data on the effect of 11,17-oxysteroid hormones in the dog are meager (Thorn, *et al.*, 1941; Davis, *et al.*, 1951; Roberts and Pitts, 1952). The latter studies were with cortisone and observations with ACTH are necessary before adequate evaluation of the relation of the ACTH-adrenocortical system to salt metabolism can be made. An important consideration which has been neglected in most studies with ACTH and cortisone in the dog and rat is the role of glomerular filtration rate in the regulation of salt and water excretion. As demonstrated in this report, data on alterations in the rate of glomerular filtration are essential for satisfactory evaluation of electrolyte excretion during hormone administration.

The present study was undertaken to help clarify the relation of adrenocortical hormones to salt and water metabolism. The salt retaining effects of ACTH, cortisone, and DCA have been compared and the relation of glomerular filtration rate to changes in electrolyte excretion and water

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exchange has been studied. Since the response to adrenocortical hormones differs among species, the results in the dog have been compared with those obtained previously in man (Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952) and rat (Rice and Richter, 1943; Ingle, *et al.*, 1946; Bergner and Deane, 1948; Boss, *et al.*, 1950).

MATERIALS AND METHODS

ACTH, cortisone, and DCA were administered to four normal female mongrel dogs. After a 2 to 3 week control period, these hormones were given successively for intervals of 5 to 31 days; a recovery period was allowed following each hormone injection period. A saline solution of ACTH[1] was injected intramuscularly every six hours whereas saline suspensions of cortisone acetate and DCA in oil were given intramuscularly once daily. Approximate doses of 2.1 and 6.3 mgm./kg./day of ACTH and 2.1 and 4.2 mgm./kg./day of cortisone were employed for dogs 1 and 2, but dogs 3 and 4 received the larger dose only (Figures 1–4). All four animals received approximately 1.0 mgm./kg./day of DCA. Two levels of sodium (Na) intake (0.5 and 3.3 m.Eq./kg./day) were given during cortisone administration to dog 3 and during injection of all three hormones in dog 4; otherwise, observations were made during the higher level of Na intake.

Glomerular filtration rate (GFR) and effective renal plasma flow (RPF) were determined in the postabsorptive state by measuring the renal clearances of creatinine (C_{CR}) and para-aminohippurate (C_{PAH}) respectively. Renal clearances were measured from 3 to 5 hours after hormone administration. During the renal function studies, blood was drawn for determination of plasma electrolytes and arterial plasma carbon dioxide content. T-1824 dye space was measured and circulating eosinophils enumerated. Femoral arterial blood pressure was determined following the renal clearance procedure.

The dogs were kept in metabolic cages except during experimental procedures. Sodium, potassium (K), chloride (Cl), nitrogen (N), and water balance studies were made according to methods reported previously (Davis and coworkers, 1952). A synthetic diet consisting of 80 Cal./kg./day was force fed so that intake was constant except during vomiting or parenteral administration of additional electrolytes. Water was allowed *ad libitum*.

The experimental procedures and chemical methods have been described elsewhere (Davis, *et al.*, 1952) with these exceptions: (1) plasma Cl in dogs 3 and 4 and the Cl content of food and feces were determined by a modified Volhard technique, (2) the carbon dioxide content of arterial plasma was measured manometrically by the method of Van Slyke and Neill (1924), and (3) for the eosinophil counts, a mixture of propylene glycol and phloxine was used as a diluent (Speirs and Meyer, 1949).

RESULTS

Alterations in renal hemodynamic function were qualitatively similar for all three hormones (Figs. 1–3). The response consisted of an increase in GFR and RPF while filtration fraction (FF) fell. Renal blood flow was also elevated since hematocrit remained at the control level. The magnitude of the effect varied considerably among animals as well as with the

[1] The ACTH was the ACTHAR product of Armour Laboratories; each unit was equivalent to 1 mgm. of the lyophilized standard LA-1-A.

hormone administered. The response was greatest with cortisone, least with DCA, and moderate with ACTH. The most marked elevations occurred in dogs 1 (Fig. 1) and 3 in which renal function increased after a single injection of each hormone. The least response resulted in dog 2 (Fig. 2) in which renal function was not detectably altered during ACTH

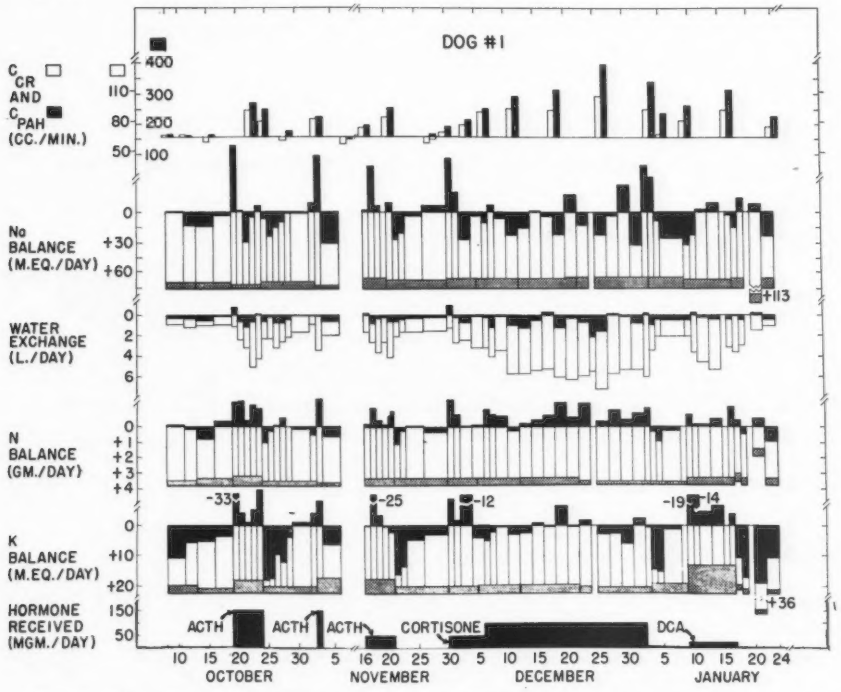

FIG. 1. The effect of ACTH, cortisone, and DCA on renal function and metabolic balances in a 24 kg. dog. Values for C_{CR} and C_{PAH} were plotted as open and solid columns respectively from a horizontal line which represents the control level. Each value is the average of 3 postabsorptive clearance periods. The convention for the balances is as follows: (1) intake was plotted downward from the zero line, (2) output was plotted upward from intake line, (3) solid black areas represent total balances, and (4) the cross-hatched areas show fecal excretion. Consequently, solid black area above the zero line indicates negative balance and black area below the zero line represents positive balance.

administration, and a slight elevation occurred only after several days of cortisone or DCA. In dog 4, GFR and RPF were increased after a single injection of ACTH and cortisone; no apparent difference was observed between the effect on low and high Na intake. DCA failed to increase renal function in dog 4 even after prolonged administration on a high Na intake.

Another striking effect of these hormones was the increase in water exchange. The typical response (3 of 4 dogs) to ACTH and cortisone was an increase in water turnover from about ½ to approximately 2 L./day (Figs. 2 and 3) but in dog 1 water exchange reached 6 to 7 L./day (Fig. 1). In this dog, the magnitude of water turnover showed a high positive

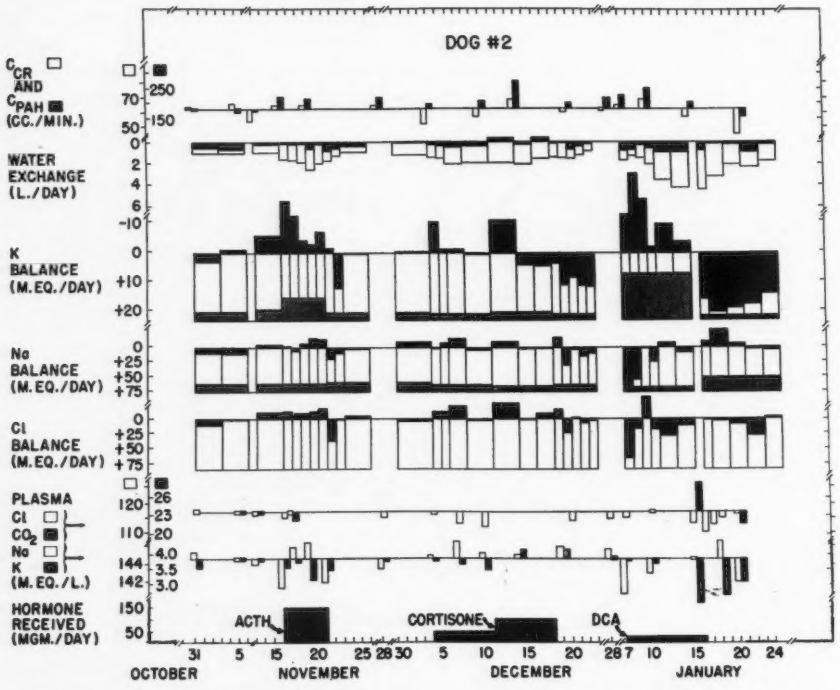

FIG. 2. Alterations in renal function, water exchange, electrolyte balances, and plasma electrolytes during ACTH, cortisone, and DCA administration to a 24 kg. dog. The horizontal line from which values for plasma electrolytes are plotted represents the average control value. See Figure 1 for the convention for the balance plots and other symbols.

correlation with the level of GFR for all three hormones and the onset of polyuria preceded the development of polydipsia. The response to DCA was less variable among the animals and a diabetes insipidus-like state occurred in all cases on a high salt diet (Figs. 1–3). The level of water exchange was independent of Na intake during cortisone administration (cases 3 and 4; see Fig. 3), but DCA had no influence on water turnover on a low Na intake (dog 4) (Fig. 3). The effect of Na intake on water exchange during ACTH administration was equivocal (Fig. 3).

The first 1 or 2 days of ACTH and cortisone injections were accompanied by salt loss in dogs 1, 3, and 4; GFR was elevated initially in these three dogs but not in dog 2 (Figs. 1–3). Salt retention resulted during the corresponding period on DCA. Thereafter, Na and Cl balance was maintained on ACTH, cortisone, and DCA although wide daily fluctuations in

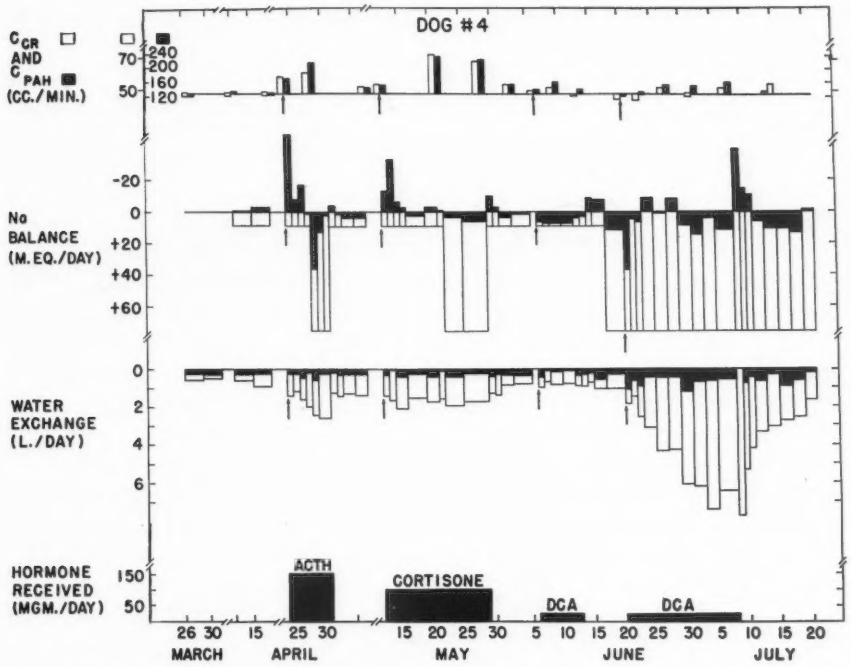

FIG. 3. Renal function, Na balance, and water exchange in a 22 kg. dog given ACTH, cortisone, and DCA during a low and a high Na intake. For a description of symbols and convention for the balances, see Figure 1.

Na and Cl excretion occurred during prolonged cortisone administration (Fig. 1). Fecal Na excretion was unaffected by ACTH and cortisone but DCA effected a slight depression which was followed by an elevation during the recovery period in cases 2 (Fig. 2) and 3.

Urinary K excretion increased initially with all three hormones during high salt intake; later, the influence of cortisone became negligible and K loss during ACTH and DCA diminished (Figs. 1, 2, and 4). In dog 4, the initial loss of K also occurred on a low salt diet with ACTH, but cortisone and DCA failed to produce a negative K balance unless salt intake was high. The greatest increase in fecal K output accompanied DCA but

an elevation also occurred with ACTH except in case 3; cortisone was without effect.

Negative K balance was most marked with DCA which resulted in a metabolic alkalosis in all instances except during low salt intake in dog 4 (Figs. 1, 2, and 4). After 8 to 10 days of DCA injections, dogs 1 and 2 exhibited signs of K deficiency including generalized muscle weakness, nystagmus, lassitude, abdominal distention, and vomiting. In contrast,

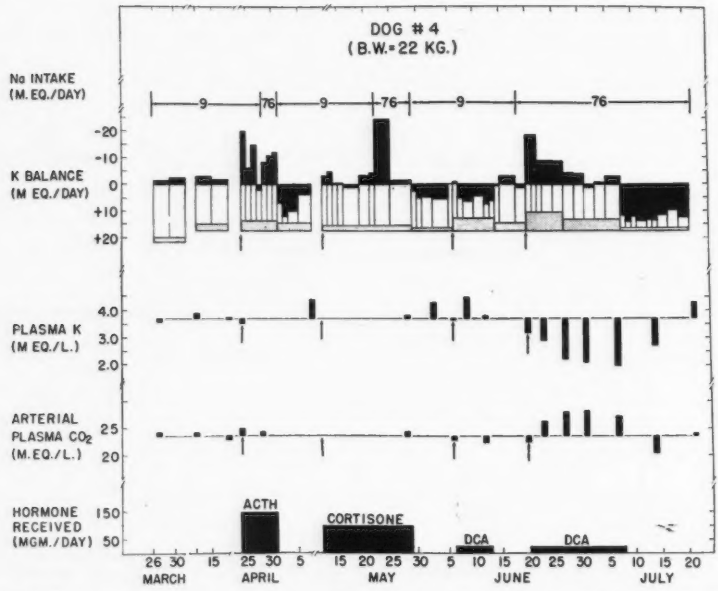

FIG. 4. Potassium balance, plasma K, and arterial plasma CO_2 during ACTH, cortisone, and DCA administration on a low and a high Na diet. Values for plasma K and arterial plasma CO_2 were plotted from a horizontal line which represents the control level. The convention for K balance has been given with Figure 1.

dog 4 was given DCA for 18 days without clinical evidence of K deficiency although plasma K dropped to 1.9 m.Eq./L. (Fig. 4). Plasma K and water exchange became progressively more abnormal as long as DCA was given, but in dog 4 plasma CO_2 reached a maximum after 11 days and remained unchanged until DCA was discontinued. Hypochloremia occurred during the DCA alkalosis in dogs 1–3 (see Fig. 2) but not in dog 4. Plasma Na was elevated during DCA administration in all but dog 2. ACTH and cortisone did not detectably alter plasma electrolytes.

A negative nitrogen balance occurred throughout ACTH and cortisone administration but the only consistent effect of DCA was increased N ex-

cretion on the first day of injection. Circulating eosinophils decreased or disappeared during injection of ACTH and cortisone in dogs 1, 2, and 4; in dog 3, repeated studies before and after ACTH administration failed to reveal a decline in eosinophils and the effect of cortisone was less than in the other cases. T-1824 dye space, hematocrit, plasma proteins, and femoral arterial blood pressure were not detectably changed. Heart rate increased only during the alkalosis with DCA.

DISCUSSION

The typical response in the renal circulation to ACTH, cortisone, and DCA was an increase in the flow of blood through the kidney. Renal arteriolar dilatation was indicated by the finding of elevated renal blood flow while arterial pressure remained at the control level. The response in renal function was observed from 3 to 5 hours after a single injection for all three hormones in dogs 1 and 3. This observation may indicate that the initial rise in renal function was effected by an action on the renal arterioles. The sustained elevation in renal function may also have resulted in part from a direct renal effect. On the basis of available data, the response to ACTH and cortisone cannot be related to expansion of the extracellular fluid volume as has been suggested for DCA by Gaudino and Levitt (1949) since the initial effect of ACTH and cortisone was a natriuresis and balances for salt and water were never positive. However, the possibility that ACTH and cortisone can effect a shift in fluid from the intracellular to the extracellular compartment as reported by Levitt and Bader (1951) has not been excluded.

The pattern of renal functional changes in the dog differs slightly from that found for man (Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951). In both species, GFR and RPF may be elevated by ACTH and cortisone but in man FF is usually increased whereas in the dog it is reduced. In the rat, no significant alteration in GFR was noted during injection of cortisone (Cotlove, unpublished data), but DCA has been reported to decrease GFR slightly (Boss, *et al.*, 1950); these studies were not accompanied by measurements of RPF.

Water exchange was increased by all three hormones but the phenomenon produced by DCA appeared to differ from that with ACTH and cortisone both quantitatively and qualitatively. A diabetes insipidus-like state occurred with DCA only, except in dog 1 in which water exchange was markedly elevated with both ACTH and cortisone. In this animal the high positive correlation between the level of GFR and water exchange may indicate that GFR played a role in the development of the diabetes insipidus-like state. Also, in dog 1 the primary polyuria during both ACTH and cortisone administration may be a reflection of the initial eleva-

tion in GFR. During injection of cortisone, water turnover was unaffected by changing the level of Na intake, whereas, as shown previously by Mulinos and associates (1941) for the dog and by Rice and Richter (1943) for the rat, the level of water exchange during DCA is a function of salt intake. In man, the level of water turnover is uninfluenced by ACTH, cortisone, or DCA (Prunty, *et al.*, 1948; Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Sprague, *et al.*, 1950; Alexander, *et al.*, 1951; Sjögren, 1952).

Distortion of the plasma electrolyte pattern and development of alkalosis which frequently results during ACTH or cortisone administration in man (Prunty, *et al.*, 1948; Sprague, *et al.*, 1950; Sjögren, 1952) failed to occur in the dog. The duration of ACTH administration may have been too short to result in alkalosis, but the dose and duration of cortisone injection were sufficient to demonstrate a negative result. The negative K balance which occurred during ACTH and cortisone was probably a reflection of increased tissue catabolism since a concomitant loss of N resulted. The data indicate that the marked K depletion which accompanied DCA resulted from a different mechanism. It seems unlikely that K loss and alkalosis during DCA administration were a passive consequence of Na retention as has been proposed by Seldin and coworkers (1951) since Na retention lasted from 1 to 2 days only whereas a negative K balance was present throughout DCA administration. A more plausible explanation is that K depletion was the primary event. Under this circumstance, it has been suggested by Cooke, *et al.* (1952), that hydrogen ions move intracellularly with Na in exchange for K and result in alkalosis. Also, K ions may be less available for distal tubular exchange for Na than normally so that sufficient hydrogen ion secretion by the kidney occurs to prevent disappearance of the alkalosis (Berliner, Kennedy, and Orloff, 1951). Failure of alkalosis to develop with DCA during low salt intake has been reported previously by Seldin and associates (1951) and is apparently related to inadequate anion excretion. Excessive loss of K by the fecal route may indicate a direct influence of adrenocortical hormones on the intestinal mucosal transport of electrolytes (Dennis and Wood, 1940).

The effect of adrenocortical hormones on salt excretion also varies among species. An initial natriuretic and chloruretic action has been reported during administration of ACTH to man (Forsham, *et al.*, 1948) and rat (Ingle and associates, 1946; Bergner and Deane, 1948), but the salt retention which follows the initial period in man has not been observed in the rat. In the present study in the dog, both ACTH and cortisone produced an initial salt loss and, like the effect in the rat, Na retention did not occur later. It has been suggested that the negative balances for Na and Cl in man resulted from a temporary increase in 11,17-oxysteroid secretion

before the effect of DCA-like hormones became manifest (Forsham, *et al.*, 1948). In the dog, it seems more likely that salt loss resulted from elevated GFR; the natriuresis occurred only in the three dogs with an initially increased GFR and not in the other animal in which GFR remained at the control level.

In contrast to the initial effect of ACTH and cortisone on salt excretion in the dog, DCA resulted in Na retention in spite of elevated GFR in two of the four dogs. This finding indicates that the Na retaining effect of DCA is greater than that of cortisone or adrenocortical hormones released by ACTH at the dosage levels employed. The effect of DCA on electrolyte metabolism appears to be similar in dog and man except that negative Na and Cl balances have been reported in man following prolonged (34–60 days) administration (Sjögren, 1952).

After the initial response, salt balance was maintained during administration of all three hormones. GFR was frequently elevated so that increased tubular reabsorption of Na and Cl can be inferred in these instances. It is not certain from these data whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules or if salt balance was achieved by another mechanism. A salt retaining effect of cortisone in the normal dog was likewise equivocal. In the adrenalectomized dog, however, cortisone appears to have a direct renal tubular action to conserve Na. Roberts and Pitts (1952) found a marked reduction in the renal excretion of Na while GFR remained at the control level. Also, adrenalectomized dogs have been maintained in excellent health for several weeks on cortisone alone (Swingle, *et al.*, 1952; Howell and Davis, unpublished data) although a low Na and a high K concentration in the plasma frequently occurred even on high doses of cortisone. The finding that a low plasma Na was associated with a negative Na balance (Howell and Davis, unpublished observations) and the present comparative data with DCA suggest that cortisone is a weak Na retaining compound. If ACTH stimulated secretion of adrenocortical salt retaining hormones, failure of salt retention might be an indication that their weak salt retaining action was overbalanced by elevated GFR.

It is evident from the foregoing discussion that the role of the ACTH-adrenocortical system in the regulation of salt metabolism has not been clearly defined. In man, it seems fairly certain that ACTH stimulates secretion of adrenocortical hormones which influence the renal tubular transport of electrolytes (Ingbar and associates, 1951), but in the dog and rat, the situation may differ. In addition to the observations with exogenous ACTH, studies during salt restriction have demonstrated that the response in hypophysectomized dogs differs from that in patients with panhypopituitarism. These patients show impaired ability to conserve

Na; in the report of Stephens (1940), a low plasma Cl (Na not measured) and evidence of acute adrenocortical insufficiency developed within three days. In contrast, the hypophysectomized dogs studied by Earle and coworkers (1951) failed to show a drop in plasma Na during ingestion of a 1 to 2 m.Eq./day Na diet for three weeks. More recently, Rolf and associates (1952) reported that hypophysectomized dogs on a low Na intake lost slightly more Na than normal dogs, but 2 to 5 weeks were required before plasma Na fell. These experiments and studies with exogenous ACTH indicate that anterior pituitary control of adrenocortical salt regulating function differs in dog and man. Additional observations of electrolyte metabolsim in the hypophysectomized dog are needed to determine the exact nature of this difference. Cytochemical studies of the adrenal cortex in the dog under circumstances similar to those reported for the rat (Deane, 1949) may also help to clarify this important aspect of anterior pituitary and adrenocortical physiology.

SUMMARY

The response to ACTH, cortisone, and DCA was studied successively for variable periods in four female mongrel dogs. GFR and RPF increased with all three hormones (except with ACTH in one dog and DCA in another). The immediate effect of these hormones may be a reflection of an action on the renal arterioles. Water exchange was increased by all three preparations. The diabetes insipidus-like state produced by DCA was dependent upon a high Na intake, whereas increased water turnover of lesser magnitude occurred with ACTH and cortisone on both low and high Na diets. A negative K balance resulted with ACTH, cortisone, and DCA but marked K depletion and alkalosis accompanied DCA and a high salt intake only. Fecal K excretion was increased greatly by DCA and slightly by ACTH (3 of 4 dogs) but cortisone was without effect. The first 1 or 2 days of ACTH and cortisone administration were accompanied by salt loss in 3 of the 4 dogs; since GFR was initially increased in these three but not in the fourth animal, a causal relationship between elevated GFR and the negative balance is suggested. Salt retention resulted during the corresponding period on DCA. Thereafter, balance was maintained during administration of all three hormones in spite of a high GFR. Although it is uncertain whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules, the effect of ACTH and cortisone on salt excretion in the dog differs from that reported for man.

Acknowledgment

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STUDIES ON THE INTERRELATIONSHIPS OF THE ADRENAL CORTEX AND RENAL AMMONIA EXCRETION BY THE RAT[1]

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IT HAS been demonstrated recently that the renal capacity of the adrenalectomized animal to secrete ammonium and hydrogen ions is deficient under normal conditions and under conditions of moderate to severe acid stress (Sartorius *et al.*, 1952). Failure to salvage adequately fixed base from the tubular urine in exchange for hydrogen or ammonium ions, results not only in increased loss of base but in a disturbance of body acid base balance. These facts probably account for the chronic acidosis and the poor tolerance to acid stress commonly exhibited by animals with adrenal insufficiency.

The present study was undertaken (a) to determine the capacity of normal and adrenalectomized animals to excrete ammonia and titratable acid when acutely subjected to mild and severe acid stress, (b) to assess the effects of desoxycorticosterone acetate and adrenal cortical extract on ammonia and titratable acid excretion by the adrenalectomized and normal rat, (c) to study the early excretory rates of these ion species after adrenalectomy in an effort to separate or to further link the renal tubular exchange mechanisms, and (d) to ascertain whether acidosis stimulates the adrenal cortex directly as evidenced by a lowering of the glandular content of cholesterol and of ascorbic acid.

METHODS

All experiments were performed on female Sprague Dawley rats weighing from 200 to 250 grams. Animals were bilaterally adrenalectomized in one stage and were allowed a recovery period of 7 to 10 days or more, except where otherwise specified. The operative procedure for the sham operated rats was the same as for those adrenalectomized except that following exposure and mobilization the glands were replaced to their normal position. Adrenalectomized rats were maintained on a high salt diet without further supporting therapy. During the course of each experiment, animals were kept in wire mesh metabolism cages, so constructed as to prevent contamination of the urine with food or feces. In all acute experiments urine was expressed from the bladder by slight supra-

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pubic pressure and collected under oil in small-necked containers in order to reduce evaporating surface. Adequate urine flows were obtained in acute experiments by the administration of 2 cc. of distilled water per 100 grams of body weight 15 minutes prior to the experiment. Water or ammonium chloride as a 5% solution was administered by stomach tube. Desoxycorticosterone acetate in sesame oil was injected subcutaneously in dosages of a half a milligram per rat, 18 hours prior to and 1 hour prior to the experiment.

Urine pH was determined with a Cambridge Glass Electrode, titratable acid by electrometric titration of the urine to a pH of 7.40, urinary ammonia by Conway's microdiffusion method (1942), adrenal cholesterol according to the method of Schoenheimer and Sperry (1934), and adrenal ascorbic acid by the method of Roe and Kuether (1943).

RESULTS

In order to quantitate more precisely the relative deficiencies in capacity of the adrenalectomized rat to secrete ammonia and titratable acid, acute experiments were performed on normal, 2 weeks adrenalectomized, and 2 months adrenalectomized rats subjected to mild and severe acidosis. Production of a mild dilution acidosis was accomplished by the administration of water alone in dosage of 2 cc. per 100 grams of body weight. A more severe acidosis was effected by the administration of ammonium chloride in dosage of 100 mg. per 100 grams of body weight dissolved in the same volume of fluid. Following an initial control period of one hour duration the acid load was imposed and the urine collected for four successive hourly periods. The numbers of rats used in any experiment are indicated in parentheses on each chart.

As is evident in Figure 1, normal rats excreted approximately 11.6 microequivalents of ammonia per 100 grams of rat per hour during the control period. With administration of water alone and the consequent production of a mild dilution acidosis, ammonia excretion rose gradually to a peak of 16 microequivalents. With the added stimulus of an acidifying agent, the excretory rate rose sharply, reaching an average level of 74.3 microequivalents for the fifth hour. In sharp contrast with these levels are those of the adrenalectomized animals. From initial control levels of but 6.6 microequivalents of ammonia per 100 grams of rat per hour, there occurred an increase to but 8.9 microequivalents under water loading and a rise to only 41.1 microequivalents with acid loading. It is thus apparent that although the adrenalectomized rat is capable of increasing ammonia output under stimulation, there is a marked deficiency in its capacity to do so. That this deficiency is not dependent upon, or altered by the length of time following operation is suggested by the fact that two weeks post-adrenalectomized rats and the same group six weeks later exhibited practically identical excretory rates for both ammonia and titratable acid.

Titratable acid excretory rates for both normal and adrenalectomized

rats are shown in the upper right hand portion of Figure 1. From control rates of less than 0.2 microequivalent excreted per 100 grams of rat per hour, normal rats responded with an increase to 4.1 micrcequivalents when given a water load and to an average level of 24.4 microequivalents in the

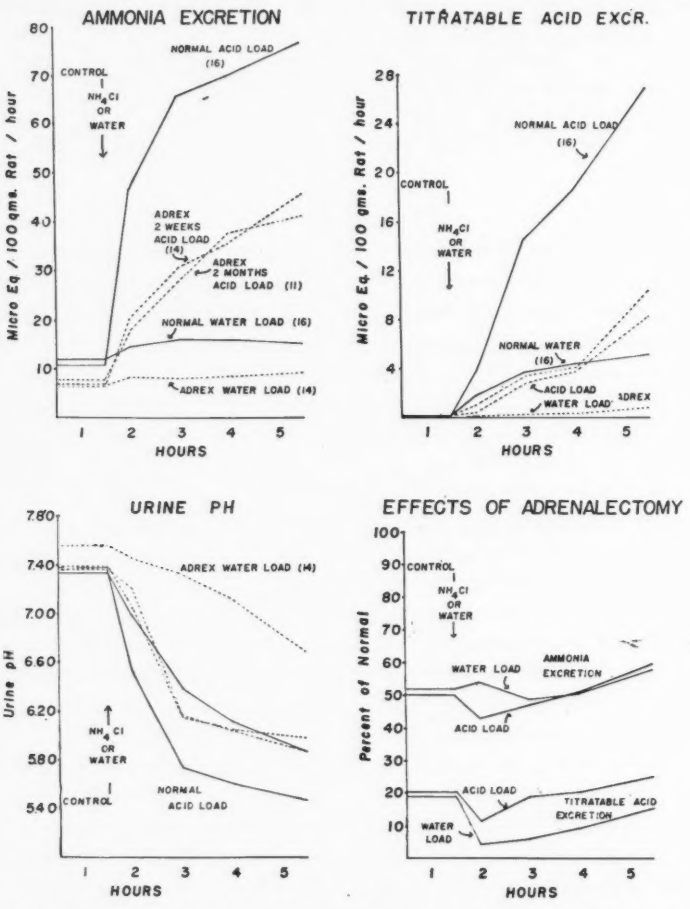

FIG. 1. The effects of adrenalectomy on the capacity of the acidotic rat to secrete ammonia and titratable acid.

fifth hour when acid loaded. A marked deficiency in the capacity of the adrenalectomized rat to secrete hydrogen ions is apparent, for the maximum excretion with water loading was 0.64 microequivalent while with the imposition of an acid load an excretory rate of only 8.4 microequivalents was attained. The higher urinary pH of the adrenalectomized rat likewise reflects this defect.

In the lower right hand corner of Figure 1, the deficiency in capacity of the adrenalectomized rat to produce ammonia and titratable acid is made more apparent by plotting the output of these substances as a per cent of normal. It is quite evident that the adrenalectomized rat is capable of pro-

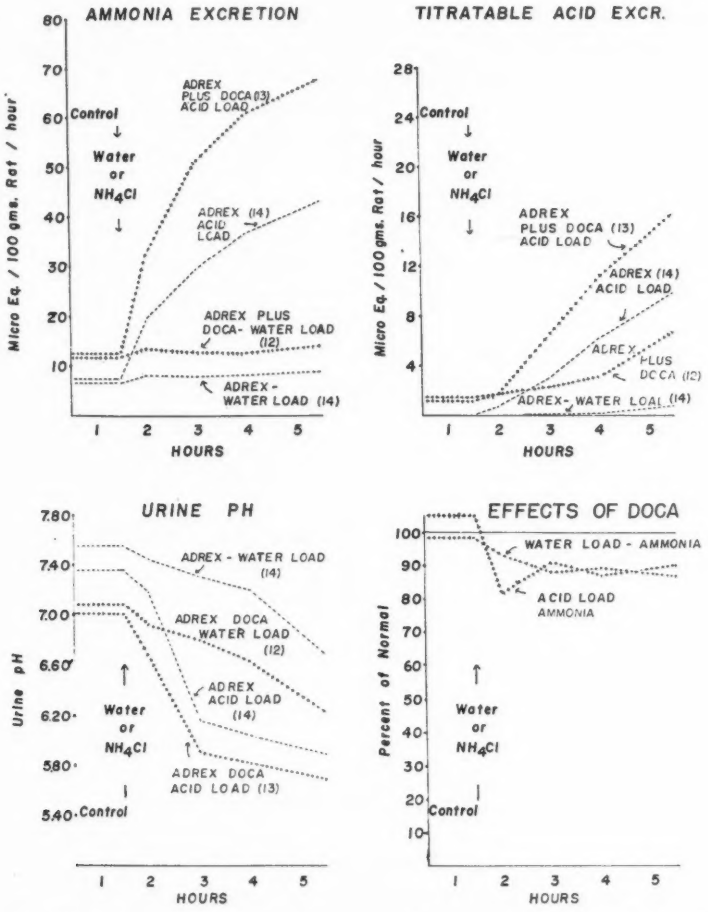

FIG. 2. The effect of desoxycorticosterone acetate on the capacity of the acidotic and adrenalectomized rat to secrete ammonia and titratable acid.

ducing ammonia at a rate of only 50–60% of that of the normal animal, and that this deficiency remains relatively constant for both control and acidotic periods. The capacity of the adrenalectomized rat to form titratable acid is even more limited, amounting to but 15 to 25% of that of the normal animal.

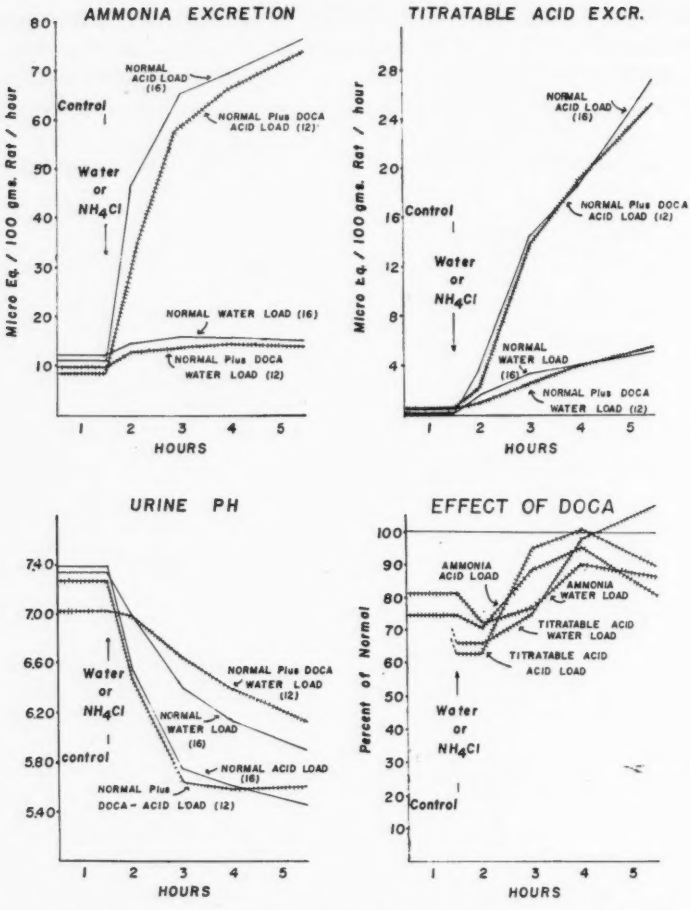

FIG. 3. The effects of desoxycorticosterone acetate on the capacity of the normal but acidotic rat to secrete ammonia and titratable acid.

Since an approximate quantitation of the reduced capacity of the adrenalectomized rat to secrete ammonium and hydrogen ions proved feasible by the administration of water or an acidifying agent in acute experiments, this technique was followed in assessing the activity of desoxycorticosterone acetate on the output of these ions species. Accordingly desoxycorticosterone was administered subcutaneously in dosage of 0.5 mg. per rat 18 hours prior to and again 1 hour prior to each experiment. The effects of the administration of his hormone to adrenalectomized rats are shown in Figure 2. It is apparent that desoxycorticosterone increased appreciably

the renal output of ammonia and titratable acid, in the control periods, and in the presence of a mild or severe acidosis. The similarity of the excretory rates of these ions by the adrenalectomized rat receiving desoxycorticosterone with the rates of the normal rat as shown in Figure 1 is striking. The fact that there is almost complete restoration to the normal is seen in the lower portion of Figure 2 where it is apparent that the adrenalectomized rats receiving desoxycorticosterone have increased their capacity to secrete ammonia some 40% or to within 90 to 100% of the normal. Titratable acid is not represented on this chart since desoxycorticosterone increased the control rate of excretion several fold in comparison with the normal. However, the restoration of acid secretory function, though considerable, is not so complete as that of ammonia secretion.

With administration of desoxycorticosterone to the normal rat no increase in ammonia or titratable acid output is apparent. Indeed, almost identical excretory rates were obtained (Fig. 3). In two additional experiments on adrenalectomized and normal rats, adrenal cortical extract in dosage of 1 cc. per rat was administered subcutaneously 18 hours prior to and again 1 hour prior to the start of the experiment. No significant difference was found between the effects of adrenal cortical extract and desoxycorticosterone on ammonia and titratable acid excretion.

Inasmuch as 1) the adrenalectomized animal exhibited a greater capacity to secrete ammonia than hydrogen ions, and 2) desoxycorticosterone acetate or adrenal cortical extract was found capable of restoring these capacities to normal, it seemed profitable to investigate the temporal appearance of these deficiencies after acute bilateral adrenalectomy. Accordingly, experiments were performed in which all animals were either sham operated or adrenalectomized after the collection of two control periods. At the time of the operative procedure the animals were given 2 cc. of water per 100 grams body weight or ammonium chloride in dosage of 1 mg. per gram of body weight in a similar amount of water. In those experiments involving the administration of an acid load, a similar dose of ammonium chloride was administered 42 hours prior to, 24 hours prior to, and 18 hours prior to the start of the experiment. Following the operative procedure, urine was collected at 2 hour intervals for 12 successive collection periods.

It is apparent that ammonia and titratable acid excretory rates for both the sham operated and adrenalectomized animals rose sharply following the administration of an acid load (Fig. 4). The adrenalectomized rats, however, attained peak excretory rates at an earlier time and at lower values than did the intact rats. As was expected, the deficiency in capacity of the adrenalectomized rat to excrete titratable acid was of greater magnitude than the deficiency in capacity to excrete ammonia. With administration of a water load, similar deficienceis were noted.

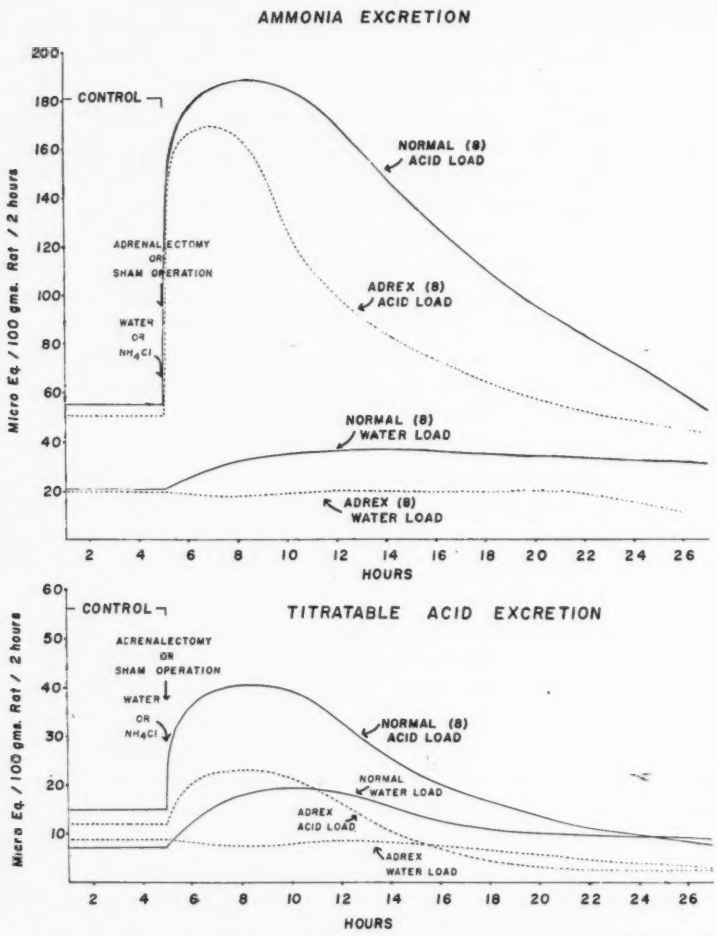

FIG. 4. The early time courses in the capacities of the sham-operated normal and acutely adrenalectomized rat to secrete ammonia and titratable acid.

It remained to be demonstrated that ammonium chloride actually stimulates the adrenal cortex. Evidence for stimulation of the adrenal cortex was obtained by administering ammonium chloride and then periodically assaying the adrenal cortices for their content of cholesterol and ascorbic acid. Urine collections were obtained at similar time intervals in order to compare the time of peak ammonia and titratable acid outputs with the time of minimum glandular cholesterol content.

As may be seen in Figure 5, the average cholesterol content fell from a

control level of 0.778 mg. per 100 grams of body weight to a low of 0.514 mg. four hours after the administration of 1 mg. of ammonium chloride per gram of body weight. Peak ammonia and titratable acid excretion occurred six hours after the administration of the acid load. Ascorbic acid content of the adrenal gland fell from control values of 5.5 mg. per 100 grams body weight to a low of 4.54 mg. two hours after the acid loading and then gradually rose to somewhat elevated values.

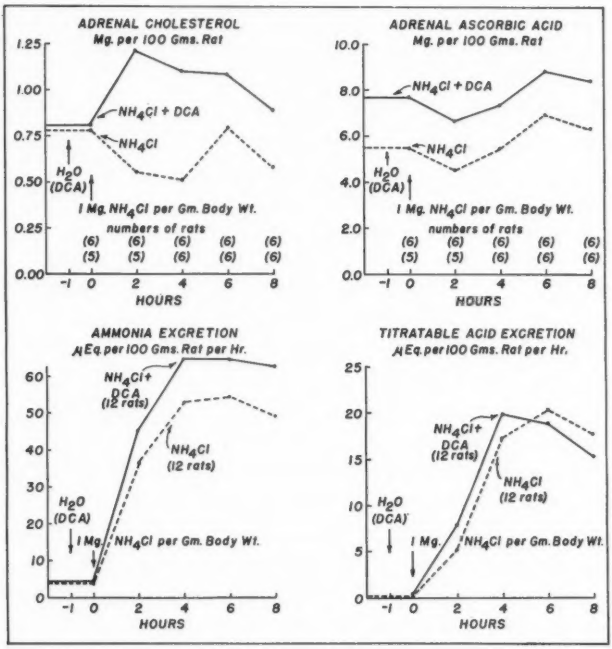

FIG. 5. The effects of ammonium chloride and ammonium chloride plus desoxycorticosterone acetate on the adrenal cholesterol and ascorbic acid content of the rat.

DISCUSSION

It is well established that the untreated adrenalectomized animal loses excessive quantities of sodium and chloride in the urine and in consequence suffers reduction in the plasma concentrations of these ions and in the volume of extracellular fluid as well (Loeb, 1932; Harrop *et al.*, 1933, 1936). However, an additional quantity of base is lost because of a relative deficiency in renal capacity to secrete hydrogen ions and ammonia (Jiminez-Diaz, 1936; Sartorius *et al.*, 1952). This deficiency accounts for the mild to moderate acidosis (reduction in bicarbonate bound base) of the ad-

renalectomized animal sustained in otherwise good condition by the administration of increased quantities of salt. The deficit in ammonia excretory capacity is roughly 50% and in titratable acid production nearly 80% under a variety of conditions of acid loading, including the normal dietary or metabolic load, mild dilution acidosis, and doses of ammonium chloride as large as 1 mg. per gm. body weight. While the adrenalectomized animal can compensate satisfactorily for moderate acid loads if provided with adequate quantities of sodium chloride, restriction of salt intake leads to rapid decline and death (Sartorius *et al.*, 1952).

The nature of the renal defect in secretion of ammonia and titratable acid has not as yet been clearly established. The fact that salt sustained adrenalectomized animals have normal rates of glomerular filtration and renal plasma flow (Lotspeich, 1949; Roemmelt *et al.*, 1949) suggests that the defect results from some specific depression of tubular activity, not as a nonspecific consequence of renal insufficiency nor as a result of morphologic alteration. The rapid restoration of function by desoxycorticosterone acetate and adrenal cortical extract is in line with this concept. The reduced capacity of the tubules of adrenalectomized animals to exchange hydrogen ions for base, reflected in a less acid reaction of the urine and reduced output of titratable acid, might result either from a depresison of specific enzymes concerned with this process or with a more general reduction in energy available to all renal cellular systems. Because the output of ammonia normally is related to the hydrogen ion concentration of the urine (Pitts and Alexander 1945; Sartorius *et al.*, 1949), a defect in ammonia secretion could result secondarily from depression of the mechanism for exchange of hydrogen ions for base. However, Jiminez-Diaz, 1936, and Russell and Wilhelmi, 1941, have demonstrated that kidney slices from adrenalectomized animals deaminate amino acids less rapidly than do slices from normal animals, a fact which suggests a specific depression of some phase of the ammonia secretory mechanism.

Our experiments demonstrate that the onset of the deficiencies in acid and ammonia excretion following adrenalectomy is rapid, for differences between "true" and sham operated animals are apparent within the first few hours. This observation suggests that the hormones responsible for activation and maintenance of these mechanisms disappear at a rapid rate. The fact that the administration of ammonium chloride to normal animals causes a decrease in the cholesterol and ascorbic acid content of the adrenal glands, suggest that glandular activation may constitute a significant part of the homeostatic response to acid loading. However, desoxycorticosterone acetate in the dosage administered did not enhance the capacity of normal animals to eliminate acid and ammonia.

SUMMARY

The kidney of the adrenalectomized rat exhibits defects in capacity to excrete ammonia and titratable acid amounting respectively to 50% and nearly 80% of the normal. These defects are apparent under usual laboratory conditions and under conditions of mild and severe acid stress. They become apparent within a few hours following adrenalectomy and persist unchanged over the period of 2 weeks to 2 months encompassed in this study. They may be corrected by the administration of adequate doses of desoxycorticosterone acetate or whole adrenal cortical extract and probably result from some specific depression in tubular metabolic activities.

As a consequence of the depression of these tubular activities, the adrenalectomized animal maintained in otherwise good condition by high salt intake suffers mild to moderate acidosis. The normal animal subjected to acid stress gives evidence of activation of the adrenal glands.

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COMPARISON OF POTENCY OF DIFFERENT ACTH PREPARATIONS BY INTRAVENOUS AND SUBCUTANEOUS ROUTES OF INJECTION

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MANY papers that indicate the existence of multiple ACTH factors have been published. Astwood (1952) and Reinhardt, Geschwind and Li (1951) have reviewed these papers recently. Most of the eivdence for two or more factors is based on quantitative differences in potency of preparations when assayed on the one hand by the ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) after intravenous injection (one hour test) and, on the other hand, by increase in adrenal weight or histological changes after subcutaneous injection (3–4 days).

Because of the differences in experimental method described in these reports, e.g., in route of injection, duration of treatment, and end point observed, the following experiments, in which only the route of injection was varied, were carried out. Hypophysectomized rats were injected subcutaneously or intravenously with two different types of preparations of ACTH. The left adrenal gland was removed one hour and the right adrenal two hours later for determination of the ascorbic acid content. It was found first, that a 100 to 1000 times larger dosage may be required subcutaneously than intravenously to cause a significant ascorbic acid depletion and second, that there was a large difference in the potency estimates with two different types of ACTH preparations depending upon the route of injection.

METHODS

Groups of male rats from Hormone Laboratories, Chicago, approximately 40 hours after hypophysectomy were injected either intravenously (nembutal anesthesia) or subcutaneously (no anesthesia) with 0.5 ml of aqueous solution of ACTH at 3 or more appropriate dosage levels, which cause a 20–50% ascorbic acid depletion. Under ether anesthesia, at one hour after injection, the left adrenal was removed, trimmed, weighed and placed in metaphosphoric acid solution. Two hours after injection the rats were killed with chloroform and the right adrenal removed and treated as was the left adrenal. Ascorbic acid content was determined by the method of Mindlin and Butler (1938). There were at least 3 rats at each dosage level.

ACTH PREPARATIONS

Frozen hog pituitary glands were ground and extracted twice at pH 2.8 using sulfuric acid and a solvent medium of 20% ethanol. The combined

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extracts were concentrated in vacuo to a volume of about 100 ml./lb. and an equal volume of acetone added. The precipitates that formed were removed and found to have little potency. The clear 50% acetone solution was run through an IR-120 column in the acid cycle. To the acid effluent from the column with a pH of about 1.6, two volumes of anhydrous acetone were added slowly, with stirring, making the acetone concentration about 84% by volume. The gummy protein precipitate which formed, was redissolved in a minimum volume of water and poured into 20 volumes of

TABLE 1. SHOWING THE RELATIVE POTENCY OF ACID, A, AND BASIC, B, ACTH PREPARATIONS WHEN ASSAYED BY TWO ROUTES OF INJECTION

(1)	(2) Dosage to cause 20% A. A. depletion		(3)	(4)
Preparation	I.V. μg.	Subcut. μg.	Dosage ratio Subcut./I.V. from Col. (2)	Ratio of A/B from Col. (3)
E 3A	0.33	100	300	3
E 13B	0.33	33	100	
M 2160A	0.5	100	200	11.1
M 2161B	1.5	27	18	
P 327A	0.04	50	1250	10.4
D 13B	0.25	30	120	
C 12A	0.1	125	1250	6
C 15B	0.2	50	250	

acetone. The flocculent white precipitate of ACTH was removed by decantation and centrifugation and thoroughly dried with acetone. Such preparations in general are soluble at all pH's in H_2O and have a potency of 2–6 I.U./mg. They are indicated as A preparations in Table 1 and Figures 1 and 2. P 327A had a higher potency because it had been refractionated in acid acetone.

The gland residue, after the second extraction above, was suspended in ethanol in an amount that resulted in an ethanol concentration of 65–70% by volume and NaOH added until the pH (glass electrode) was 9.5–10. The third and fourth extracts were made under these conditions, which were those used by Bates and Riddle (1935) to extract prolactin from bovine pituitaries. The extracts were neutralized to pH 6 and 2 volumes of ethanol added to precipitate the ACTH and other proteins. The precipitate was then extracted twice with 50% acetone at pH 9 and the extract adjusted to pH 3 where another precipitate was removed and re-extracted. The pH 3, 50% acetone soluble fraction was then deashed by use of an IR-120 resin column as for the first and second extracts but the effluent was made up to 90–95% acetone at once. The white precipitate was dried and contained the ACTH from the third and fourth or alkaline extracts.

Such preparations are insoluble in water between pH 3.5 and 6.5 and usually have a potency of 1 to 2 I.U./mg. They are indicated as B preparations in Table 1 and Figures 1 and 2.

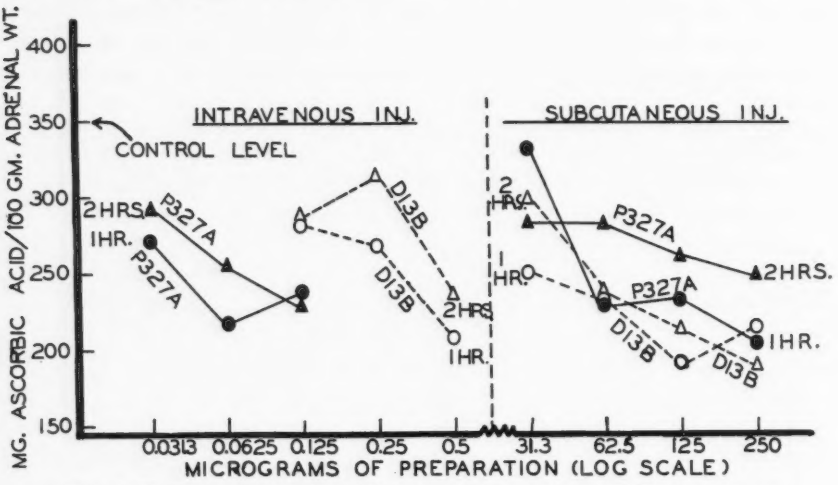

FIG. 1. Showing quantitative differences between two ACTH preparations injected by different routes.

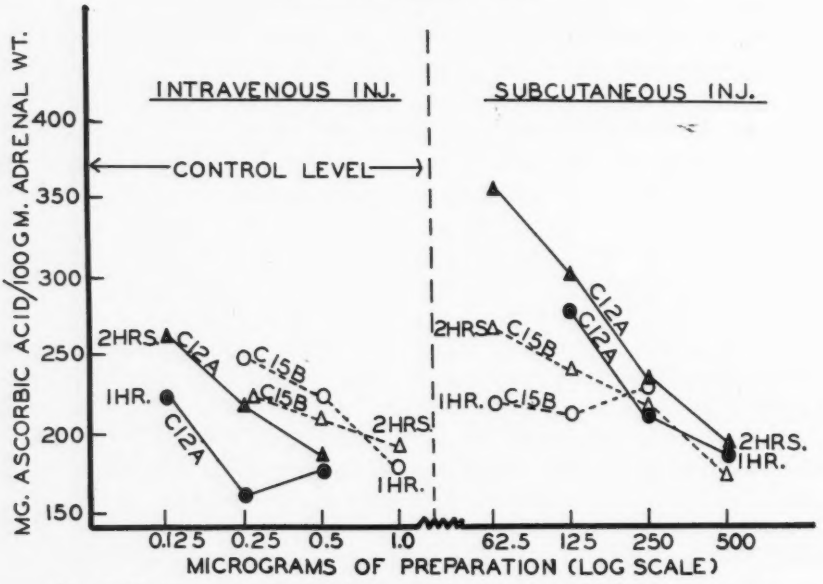

FIG. 2. Showing quantitative differences between two ACTH preparations injected by different routes.

RESULTS

Four comparisons were made between A extracts which were acid extracts and B extracts which were basic or alkaline extracts. A comparison of the effect of two pairs of these preparations upon the ascorbic acid levels in the adrenal when injected intravenously and subcutaneously are shown graphically in Figures 1–2. In both cases it can readily be seen that the A fractions are more potent than the B fractions by intravenous injection. In contrast, when given subcutaneously, the B preparations cause an equal or greater depletion of ascorbic acid than the A preparations.

Another highly significant fact shown in these figures is that the subcutaneous dosage required to cause 20–50% depletion of ascorbic acid is 100 or more times greater than the intravenous dosage. It is apparent from the figures that the slopes of the dosage response lines with different preparations are not always parallel especially when the subcutaneous route of injection is used.

In general, the ascorbic acid levels of the adrenals two hours after injection are slightly higher than after one hour indicating that the adrenal ascorbic acid level is beginning to return towards the control level. The ascorbic acid levels at the two time intervals are confirmatory of each other so that the comparisons may be considered as being carried out in duplicate. The two time intervals were used because we had previously found that the fall in ascorbic acid level after ACTH was injected intravenously was maximum in 10 to 15 minutes whereas it was not maximum until about one hour after ACTH was injected subcutaneously. It was thought desirable to consider time differences in the experiment due to the different rates of action of the ACTH by the different routes of injection.

By averaging the one and 2 hour data and by graphical interpolation and extrapolation, an estimate was made of the dose to cause a 20% ascorbic acid depletion by the different routes of injection. This is shown in column 2 of Table 1. These estimates, of course, are subject to considerable error due to the small number of animals used, the variability of the amount of depletion in different rats and the differences in slope, especially the tendency for horizontal slope with the subcutaneous route. In our laboratories 0.5 to 1 milliunit of the International Standard, when given intravenously, will cause a 20% depletion of the ascorbic acid level. Thus, preparation P 327A has a potency of 10 to 20 I.U./mg., and D 13B of 2 to 4 I.U./mg. In column 3 it is shown in the case of P 327A that 1250 times more ACTH is required subcutaneously than intravenously to cause a 20% ascorbic acid depletion whereas with D 13B only 120 times as much ACTH is required.

Column 4 shows how much the relative potency of the A and B fractions differ with the different routes of injection. Thus E 3A would be only $\frac{1}{3}$ as

potent as E 13B if the potency were determined by the subcutaneous route whereas they are of equal potency by the intravenous route. Likewise P 327A, which is approximately 6 times more potent than D 13B by intravenous assay is only 60% as potent as D 13B by subcutaneous assay making an over-all difference of potency of 10.4 times depending upon whether the intravenous or subcutaneous estimates of potency were used.

An additional experiment was run comparing intraperitoneal and subcutaneous routes of injection. The dosage ratios by the two routes expressed as in Table I, column 3, (Subcut./I.P.) were 1.1 for M 2160A and 1.33 for M 2161B, which values are not significantly different from 1.0.

A preliminary experiment with subcutaneous injection twice daily for four days of P 327A and D 13B into hypophysectomized rats indicated that they were about equal in adrenal weight increasing potency. A 50% increase in adrenal weight was obtained with 6.7 mg. and 3.3 mg. respectively. As would be expected, the adrenal weight increasing potency was more nearly correlated with the ascorbic acid depletion tests when injections were made subcutaneously than when made intravenously.

Unpublished results, using adrenal ascorbic acid depletion in normal rats as the end point, upon duration of action of single subcutaneous injections of 16 I.U. of ACTH formulated in sesame oil in a manner to slow absorption (see also Astwood, 1952), show that ACTH preparations like A and B can be made to increase adrenal weight by over 50% in 24 hours and 100% in 48 hours while maintaining ascorbic acid levels more than 50% below control levels. This remarkable increase in adrenal weight is due to the slow release of ACTH over a period of days, as shown by the ascorbic acid levels, and not to any special qualitative property of the ACTH in the preparations. Numerous clinical reports of the high potency of ACTH given by slow intravenous infusion have been made (Mandel, Singer, Gudmundson, Meister and Modern, 1951; Renold, Forsham, Maisterrana and Thorn, 1951).

DISCUSSION

Astwood and coworkers (1952) have made preparations of ACTH by adsorption on oxycellulose that are 25 to 100 times more potent than the ones used here. Hence, the preparations used here and those used by most other investigators contain 95% or more of non-ACTH protein or protein-like material. The different ascorbic acid depletion potencies of the A and B preparations, shown above, and different solubilities probably are not due to the ACTH itself but are due to differences in the 95% contaminants and their effect upon the rate of absorption from the site of injection.

Different estimates of potency by the different routes of administration are important clinically because most ACTH is used by routes of injection

other than the intravenous route which is used for standardization. Assay of ACTH preparations by the universally used intravenous Sayers or Munson methods probably does not properly indicate the clinical efficacy of the preparation when given intramuscularly. Conversely a subcutaneous assay method would not properly indicate the intravenous efficacy of a preparation. In all 4 cases in Table 1 the iso-electrically precipitable fraction, B, is much more potent subcutaneously than the intravenous assay would indicate it to be. Clinical proof that a similar difference between these preparations occurs in man is not available.

Most differences in physiological efficacy of ACTH are as readily explained on the basis of different rates of absorption, which depends upon the route of injection and the nature and formulation of the preparation, as on the basis of a qualitative difference in the ACTH present, i.e., the multiple factor theory.

SUMMARY

The potencies of two different types of ACTH preparations were compared by intravenous and subcutaneous injection using adrenal ascorbic acid depletion in the hypophysectomized rat as the criteria of potency. Estimates of the relative potency of the two types differed with the route of injection by 3 to 11 times. The subcutaneous route of injection required as much as 100 to 1000 times larger dosage than did the intravenous route to cause equivalent ascorbic acid depletion. The data given are interpreted as not supporting the multiple ACTH factor therory.

Acknowledgements

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RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE, AND TESTOSTERONE PROPIONATE COMBINED WITH GROWTH HORMONE

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IN THE human, normal growth appears to occur in two phases: prepuberal growth, hypothesized as being entirely referable to pituitary growth hormone, and puberal growth referable to steroid growth promoting factors from the gonads and the adrenal cortex (Kinsell *et al.*, 1948). Nitrogen retention and protein anabolic effects of testosterone and its derivatives, methyltestosterone and particularly testosterone propionate have received considerable attention, and, although much factual information has been obtained (Kochakian, 1946, 1950), elucidation of the mechanism of these effects is still awaited.

Technical procedures and mathematical treatment developed by Sprinson and Rittenberg (1949) and Hoberman (1949–50) for assessing protein and amino acid metabolism in terms of rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool have been utilized in characterizing and quantitating the over-all mechanisms involved in nitrogen storage induced in the adult female dog with anterior pituitary growth hormone (Bartlett and Gaebler, 1952). The effect of growth hormone in depressing the rate of amino acid catabolism and increasing the size of the nitrogen pool was particularly striking and as previously suggested (Bartlett and Glynn, 1950) perhaps indicative of the mechanism by which growth hormone regulates the intracellular synthesis of protein.

Extension of these studies to include nitrogen storage induced in the adult female dog with testosterone propionate seemed particularly desirable not only from the standpoint of comparing the pattern of the over-all mechanisms involved in the metabolism of proteins and amino acids with the pattern observed during nitrogen storage induced by growth hormone but also because of widespread clinical interest in the use of androgens to promote nitrogen retention. It was felt that information of this sort might also be of value in extending our knowledge and understanding of the role of androgens in the regulation of puberal growth. In

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view of the summation effect of testosterone propionate and growth hormone on body weight and protein anabolism of castrated mice (Kochakian and Stettner, 1948), it also seemed of interest to include experiments in which nitrogen storage was induced by the simultaneous administration of growth hormone and testosterone propionate at dose levels corresponding to those given in single experiments.

EXPERIMENTAL

Animals—Dogs 44, 52, and 55 utilized in the preceding study with growth hormone (Bartlett and Gaebler, 1952) and maintained in the manner previously described (Gaebler, Bartlett and Sweeney, 1951) were used in the experiments reported herein.

Procedure—Since data were available on the rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool for each of these animals during nitrogen balance experiments (Bartlett and Gaebler, 1952), it was only necessary to conduct experiments in which nitrogen storage was induced with testosterone propionate or the combination of testosterone propionate with growth hormone. The experimental procedure followed and the mathematical treatment of the data were essentially the same as previously described. Constancy of both body weight and total urinary nitrogen excretion were taken as indicative of nitrogen equilibrium and nitrogen storage was induced in such animals either by the intramuscular injection of testosterone propionate at dose levels of 50 mg./day for 5 days, or by a combination of this level of androgen with the subcutaneous administration of growth hormone in doses of 5 mg./day for 5 days. N^{15}-labeled glycine was administered on the 4th day of induced nitrogen storage experiments and urinary excretion of the N^{15} label determined at 24 and 48 hours after ingestion of the amino acid.

RESULTS

In Table 1 are summarized the results of experiments in which nitrogen storage was induced with testosterone propionate. The rate of protein synthesis, S_k, and the size of the nitrogen pool, P_k, were calculated according to the mathematical treatment of Sprinson and Rittenberg (1949), and, k_1 and k_2, rates of amino acid catabolism and incorporation of amino acids into protein, by the mathematical treatment of Hoberman (1949–50). S_k values previously reported (Bartlett and Gaebler, 1952) for Dogs 44, 52, and 55 during nitrogen balance experiments were respectively in contrast to those given in Table 1—0.49, 0.56, and 0.82 gm. N/kg. body wt./24 hrs., and P_k values were respectively 0.82, 0.91, and 0.95 gm. N/kg. body wt. Values of k_1 abstracted from the same reference were respectively 3.85, 3.96, and 2.74%/hr. and values for k_2 3.85, 4.19, and 3.60%/hr. In general, the rate of protein synthesis was higher and the rate of amino acid catabolism lower during nitrogen storage induced with testosterone propionate than during the nitrogen balance experiment. There appeared to be no significant change in the size of the nitrogen pool, P_k.

Results of experiments in which nitrogen storage was induced in Dogs 44 and 55 by the combination androgen and growth hormone treatment are presented in Table 2. Comparison of this data with the nitrogen balance

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF THE NITROGEN POOL

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N	
					Catabolized and excreted, k_1 (%/hr.)	Incorporated into protein k_2 (%/hr.)
44	0.31	3.89	0.65	0.82	2.18	4.70
52	0.14	2.66	0.79	0.81	3.19	5.51
55	0.43	3.40	0.66	1.05	2.38	3.52

[1] Nitrogen storage induced in Dogs 44, 52 and 55 with Schering's Oreton brand of testosterone propionate.

[2] Weight gain was computed by determining the difference between the maximum weight attained during the 5 day period of induced nitrogen storage and the weight of the animal preceding induction of nitrogen storage.

[3] Nitrogen storage was calculated from total urinary nitrogen excretion alone. The average of 5 or more days preceding induction of nitrogen storage was taken as the control level and the difference between this value and the urinary nitrogen excretion for each day of the 5 day period determined. The values given in the table are summation values for the 5 day period of storage.

data from the same animals results in a number of interesting observations. In spite of the induction of marked nitrogen storage and gain in body weight as a result of the combined treatment with androgen and growth hormone, the rate of protein synthesis calculated in terms of either S_k or k_2 was not appreciably affected. Rates of amino acid catabolism were decreased in both animals and the size of the nitrogen pool was reduced.

TABLE 2. RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM, AND SIZE OF THE NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE PLUS GROWTH HORMONE

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N	
					Catabolized and excreted, k_1 (%/hr.)	Incorporated into protein, k_2 (%/hr.)
44	0.71	10.88	0.47	0.58	1.86	3.94
55	0.79	11.18	0.74	0.87	2.28	4.30

[1] Nitrogen storage was induced in Dog 44 with testosterone propionate (Oreton) combined with growth hormone preparation Rx099916, supplied through the courtesy of Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis & Company, and, in Dog 55 with testosterone propionate (Oreton) combined with growth hormone preparation 22KR1 supplied by Armour and Company.

[2,3] Please refer to footnote of Table 1.

DISCUSSION

The observation of a decrease in the rate of amino acid catabolism, k_1, in the normal adult female dog during nitrogen storage induced with testosterone propionate is in agreement with the observation that decreased urinary urea excretion in this animal is not due to elevated blood nonprotein nitrogen (Gaebler and Tarnowski, 1943). In terms of rates of protein

synthesis and amino acid catabolism, nitrogen storage induced in the adult female dog with testosterone propionate appears to be qualitatively similar to nitrogen storage induced with growth hormone (Bartlett and Gaebler, 1952), *i.e.*, characterized by a higher rate of protein synthesis and a lower rate of amino acid catabolism than observed in the same animal during nitrogen balance. Testosterone propionate in contrast to growth hormone appears to have no significant effect on the size of the nitrogen pool.

In considering changes in the rates of the principal over-all reactions required to either increase or decrease the size of the metabolic pool of nitrogen, relative to nitrogen balance values, a decreased rate of protein synthesis or amino acid catabolism or an increased rate of protein degradation might singly or in one of several combinations result in a net increase in the size of the nitrogen pool, while an increased rate of protein synthesis or amino acid catabolism or a decreased rate of protein degradation might similarly result in a net decrease in the size of the nitrogen pool. In view of the fact that the pattern of protein metabolism in terms of rates of protein synthesis and amino acid catabolism is qualitatively similar in nitrogen storage induced by either growth hormone or testosterone propionate, the contrast in the net effect on the size of the nitrogen pool seems of special interest. The complexity of the situation and the difficulty involved in its interpretation is emphasized with the realization that the net effect on the size of the nitrogen pool could, in addition to those factors previously noted, also be determined by either the extent to which the rate of protein synthesis was increased and the rate of amino acid catabolism decreased or by changes in the rate of protein degradation or by a combination of these contributing factors. The importance and necessity of having data on the relative rates of protein degradation during both nitrogen balance and nitrogen storage experiments seems quite apparent, and until such data are available, progress in the elucidation of the mechanisms involved in nitrogen storage and growth and particularly in respect to the accumulation of protein will be limited.

The observation that growth hormone (Bartlett and Gaebler, 1952) and testosterone propionate administered alone increased the rate of protein synthesis, but when administered in combination had no significant effect on this value, and the observation that the rate of amino acid catabolism was decreased are of special interest. The fact that the size of the nitrogen pool actually decreased under these conditions suggests that the combination androgen and growth hormone treatment resulted in a significant reduction in the rate of protein degradation.

In connection with the hypothesized endocrine regulation of prepuberal and puberal growth (Kinsell *et al.*, 1948), it should be noted that growth during the puberal period, which is particularly characterized by the accumulation of body protein (*e.g.*, in the development of the musculature),

might also be referable to a combination of growth hormone and steroid growth promoting factors such as the androgens and estrogens. If such is the case, and, the results of our experiments in which nitrogen storage was induced in the adult female dog with growth hormone and in combination with testosterone propionate can be interpreted respectively in terms of simulated prepuberal and puberal growth then the latter in contrast to prepuberal growth may be largely due to a decrease in the rate of protein degradation. The relatively high protein requirements and output of nitrogen during the phase of prepuberal growth as compared with that during puberal growth might be taken as evidence supporting the view that prepuberal growth is characterized by a high level of protein metabolism, *i. .e.*, a high rate of protein synthesis and a high rate of protein degradation, whereas, puberal growth is characterized by a relatively lower level of protein metabolism, *i.e.*, relatively lower rates of protein synthesis and protein degradation.

SUMMARY

Nitrogen storage induced in the adult female dog with testosterone propionate is characterized by an elevation in the rate of protein synthesis and a decrease in the rate of amino acid catabolism relative to values observed in the same animal during nitrogen balance experiments.

Testosterone propionate had no significant effect on the size of the metabolic pool of nitrogen in the adult female dog.

A combination of growth hormone and testosterone propionate produced marked nitrogen storage and gain in body weight, a decrease in the rate of amino acid catabolism and the size of the nitrogen pool in the adult female dog. The rate of protein synthesis was unaffected in the same animal.

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ERRATA

Due to an error in calculation, the following corrections should be noted:

Ryan, K. J. and Engel, L. L. *Interconversion of Estrone and Estradiol-17 by Rat Liver Slices.* Endocrinology *52*, 277–280 (1953).

p. 278. "*Incubation:* . . . hormone concentration of 0.001, 0.0002 and 0.00006 mg. hormone/mg. liver/ml. buffer . . . a hormone concentration of 0.0002 . . . ."

Table 2 Heading of column 1 should read "Hormone concentration* $\times 10^2$."

p. 281. Last paragraph, line 7 should read "when an 0.0002 hormone . . . "

p. 282. Line 1, should read " . . . increased to 0.001 . . . . "

Table 3 Column 2. Heading should read "Hormone concentration $\times 10^2$."

p. 282. Paragraph 2, line 3 should read "hormone concentration of 0.0002 mg. hormone . . . "

Line 6 should read " . . . decreased to 0.00006 . . . "

p. 285. Paragraph 2, line 8 should read " . . . (0.00006) . . . "

THE INTERCONVERSION OF ESTRONE AND ESTRADIOL-17β BY RAT LIVER SLICES[1,2,3]

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SINCE Zondek's original demonstration (1934) that rat liver mince "inactivates" estrogens, many workers have engaged in the study of the *in vitro* metabolism of these steroids employing loss of biological activity as a means of following hormone degradation (Heller, 1940; Engel, P. and Rosenberg, 1945; Twombly and Taylor, 1942; Levy, 1947 and DeMeio *et al.*, 1948). Pearlman and DeMeio (1949) accomplished the only isolation of estrone after the incubation of estradiol-17β[5] with rat liver slices. A technique for the isolation and determination of estrogens in minute quantities would be of great assistance in studying this reaction in detail. Counter-current distribution and fluorometric analysis have provided a means for the separation, quantitative estimation and identification of natural estrogens in urinary extracts (Engel *et al.*, 1950). This report describes the application of these techniques to the analysis of estrogens after incubation with rat liver slices.

METHODS

Tissue Preparation: Female Wistar strain rats, 6–10 months old were decapitated and bled. Their livers were quickly removed, chilled, sliced to 0.5 mm. thickness with a Stadie-Riggs microtome, drained and weighed. Cirrhosis and hepatomas were induced in similar rats by the administration of a high fat diet containing p-dimethylaminoazobenzene (Kline *et al.*, 1946) and prepared for incubation as described above. When hepatomas were used for incubation, they were dissected free from surrounding tissue. Microscopic sections were prepared and examined to insure purity of the preparation.

Hormone Preparation: Estradiol was analyzed by the counter-current, fluorometric

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[1] This work was supported in part by a grant from the National Cancer Institute, U. S. Public Health Service and in part by an Institutional Grant from the American Cancer Society, Inc., to the Massachusetts General Hospital.

[2] A preliminary report of this work was presented at the Second International Congress of Biochemistry, Paris, France, July 21–26, 1952.

[3] This is publication #777 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

[5] Hereafter referred to as estradiol. Nomenclature employed is that of Fieser and Fieser (1949).

method of Engel *et al.* (1950) and found to contain less than 0.1% fluorogenic contaminants. Two solutions were prepared for use:

1. Concentrated solution in absolute ethanol (6 mg./ml.) stored at 0° C.
2. Aqueous solution prepared just prior to use by heating 5 mg. estradiol with 0.25 ml. N NaOH and diluting with 2.25 ml. distilled water to make a final concentration of 2 mg./ml.

Estrone prepared by partial synthesis from plant sources was obtained and by similar analysis contained less than 0.1% fluorogenic contaminants.[6] This material was prepared as follows:

1. Concentrated solution in absolute ethanol (2.25 mg./ml.) stored at 0° C.
2. Aqueous solution prepared as in the case of estradiol.

Both hormones were also examined by ultra-violet spectroscopy and found to be free from contaminants absorbing in the range 220–350 mμ. Paper chromatography (Heftman, 1950) showed the estradiol to be contaminated by very small amounts of dihydroequilenin.

Buffer: Krebs' phosphosaline buffer (1940) was prepared just prior to use and the pH adjusted to 7.4. In one instance, an intracellular buffer was used (Buchanan, Hastings and Nesbett, 1949).

Incubation: From 0.5 mg. to 5.0 mg. of hormone was incubated with 0.1 to 5 gm. of rat liver with resultant hormone concentrations of 0.1, 0.02 and 0.006 mg. hormone/mg. liver/ml. buffer. (Except where otherwise stated, a hormone concentration of 0.02 was used.)

In a typical experiment, 10 ml. of Krebs' phosphosaline buffer and 1 mg. of hormone (dissolved in ethanol unless otherwise stated) were placed in a 50 ml. Erlenmeyer flask. The pH was adjusted to 7.4; 0.5 gm. of sliced liver was added to each flask; the preparations were stoppered and incubated for 4 hours with constant agitation in a water bath at 37.5° C. For the aerobic experiments, air was used as the gas phase; anaerobic conditions were achieved either by bubbling nitrogen through the mixture prior to incubation or in evacuated Thunberg flasks. Incubation mixtures not extracted immediately were stored at −10° C.

Extraction: The processing of the incubation mixture is outlined in the accompanying diagram (Fig. 1). Two extraction procedures were used which differed only up to the pentane-methanol distribution. Recoveries from both methods were comparable. Method A was suggested by Segaloff.[7]

Analysis and Characterization of Tissue Extracts: The counter-current distributions of the residues from the final ether extracts were performed in the 25-tube Craig steel apparatus (Craig and Post, 1949), and analyzed fluorometrically.

RESULTS

Control Experiments: Extraction and fluorometric analysis of the hormone-liver slice preparation before incubation showed that 91% of the starting material could be recovered unchanged. All tissue incubated without hormones was extracted and analyzed fluorometrically and showed no contaminating estrogens. No interconversion was observed when estrone

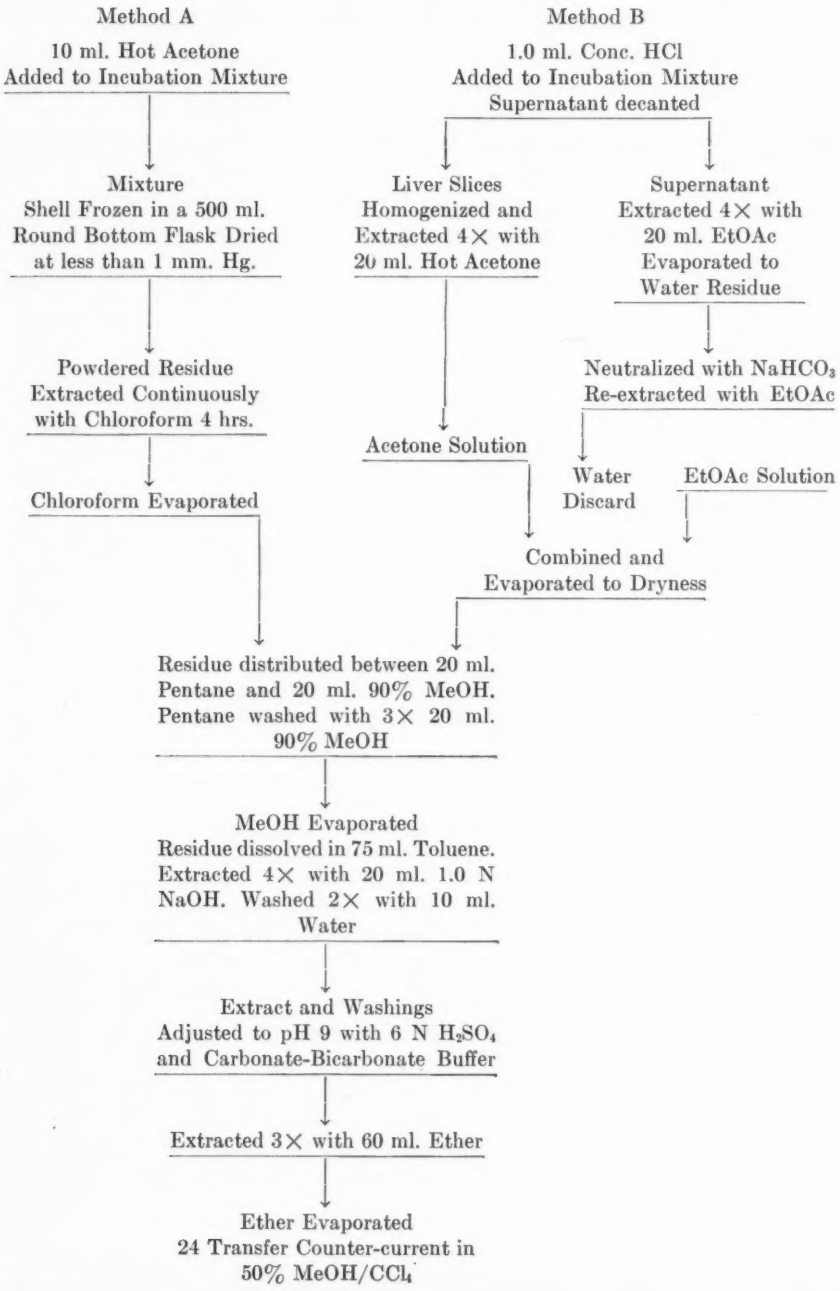
[6] A sample of estrone from animal sources was analyzed by countercurrent distribution and fluorometry and found to be contaminated with 4.6 to 5.5% estradiol.

[7] Personal communication from Dr. A. Segaloff.

FIG. 1

The Extraction of Estrogens from Tissue Incubation Mixtures

Extraction Procedure


Method A
10 ml. Hot Acetone Added to Incubation Mixture
Mixture Shell Frozen in a 500 ml. Round Bottom Flask Dried at less than 1 mm. Hg.
Powdered Residue Extracted Continuously with Chloroform 4 hrs.
Chloroform Evaporated
Method B
1.0 ml. Conc. HCl Added to Incubation Mixture Supernatant decanted
Liver Slices Homogenized and Extracted 4× with 20 ml. Hot Acetone
Acetone Solution
Supernatant Extracted 4× with 20 ml. EtOAc Evaporated to Water Residue
Neutralized with $NaHCO_3$ Re-extracted with EtOAc
Water Discard
EtOAc Solution
Combined and Evaporated to Dryness
Residue distributed between 20 ml. Pentane and 20 ml. 90% MeOH. Pentane washed with 3× 20 ml. 90% MeOH
MeOH Evaporated Residue dissolved in 75 ml. Toluene. Extracted 4× with 20 ml. 1.0 N NaOH. Washed 2× with 10 ml. Water
Extract and Washings Adjusted to pH 9 with 6 N H_2SO_4 and Carbonate-Bicarbonate Buffer
Extracted 3× with 60 ml. Ether
Ether Evaporated
24 Transfer Counter-current in 50% MeOH/CCl_4

and estradiol were incubated with boiled liver and an average recovery of 76% was obtained (Fig. 2). Owing to changes in the physical state of the tissue proteins, the extraction of estrogens from boiled liver is rendered more difficult.

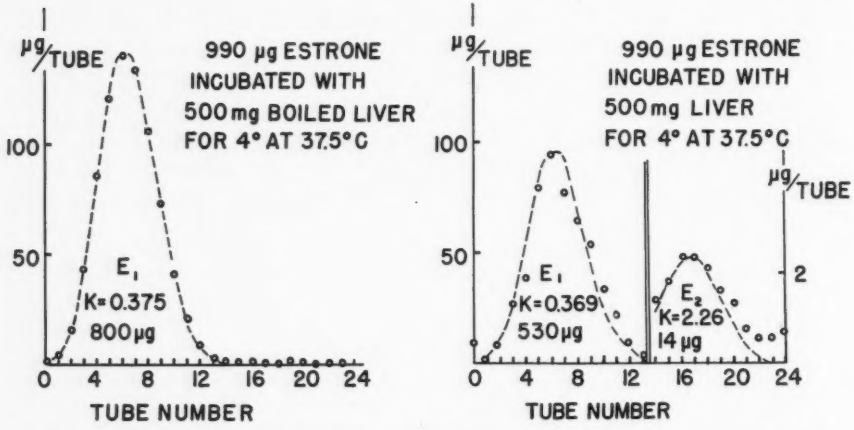


FIG. 2. Incubation of estrone with rat liver slices. Typical counter-current distribution curves. Intact liver slices converted estrone to estradiol. Boiling the tissue prevented conversion. E_1—Estrone; E_2—Estradiol; K—Partition Coefficient; ₀—Experimentally determined points; - - - —Theoretical.

TABLE 1. EFFECT OF VARYING THE HORMONE SOLVENT ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Solvent	Initial estradiol μg	Total recovery %	Estradiol recovered μg	Estradiol recovered K*	Estrone yield μg	Estrone yield %	Estrone yield K*
Aqueous	970	46%	430	2.1	18.5	1.9	0.34
	970	63	605	2.2	9.2	0.9†	0.31
	970	29	276	2.0	7.5	0.8	0.36
	490	64	294	2.1	18.2	3.7	0.39
	900	43	365	2.1	22.0	2.5	0.38
Ethanol	1020	64	555	2.1	93.0	9.1	0.33
	1020	26	222	2.1	39.8	3.9	0.36
	1020	22	188	2.3	36.1	3.5†	0.36
	1020	48	440	2.0	47.0	4.6	0.36
	1020	68	633	2.2	61.2	6.0‡	0.35
Aqueous with added Ethanol	980	37	336	2.0	21.4	2.2	0.39
	660	46	260	2.1	38.3	5.8	0.41
	750	35	233	2.1	31.0	4.2	0.38

* K—Partition coefficient in 50% MeOH—50% H_2O/CCl_4
†—2 hour incubation.
‡—Intracellular buffer.

Incubation of Estradiol with Normal Liver Slices (*Table 1*): When estradiol in ethanol solution was incubated with normal rat liver slices using a hormone concentration of 0.02 mg./mg. tissue/ml. buffer, there was an average yield of 5.4% estrone and a recovery of 40% of unchanged estradiol. This is in close agreement with the data of Pearlman and DeMeio (1949) who isolated 6% estrone and recovered 40% estradiol after incubation of the hemisuccinate of estradiol with rat liver slices. The 55% of

TABLE 2. EFFECT OF VARYING THE HORMONE CONCENTRATION ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Hormone concentration* $\times 10^2$	Initial estradiol μg	Total recovery %	Estradiol recovered		Estrone yield		
			μg	K	μg	%	K
0.1	5100	72	3630	2.2	51	1.0	0.35
	1020	45	450	2.1	14	1.4	0.37
0.02 (Aver. of 3 runs Table I)	1020	46	408		54	5.9	
0.006	1020	33	191	2.1	147	14	0.37
	1020	0.3	1.8	2.1	1.2	0.1	0.37

*—Hormone concentration = mg. hormone/mg. liver/ml. buffer.

starting material unaccounted for represents conversion of estrone and/or estradiol to unknown metabolites.

An experiment employing an intracellular buffer is listed in Table 1 but the results are not strikingly different from those obtained with the conventional Krebs' solution. Results similar to these have been reported by DeMeio *et al.* (1948).

In the experiments cited above, alcohol was used as solvent to disperse the estradiol in the buffer solution. The amount of ethanol used was similar to that employed by DeMeio (1948). To test the possible effect of the ethanol on the metabolic reaction, the sodium salt of estradiol was prepared and added to the buffer solution. When the pH was adjusted to 7.4 the hormone was precipitated as a fine dispersion. The results of using this aqueous dispersion are shown in Table 1. When an ~~0.02~~ hormone concentration was used, the average yield of estrone was 2.7% and the recovery of estradiol was 49%. If the incubations were carried out with aqueous dispersions of the steroids (prepared from the sodium salts) to which ethanol was added, the yield of estrone was increased to 4% and the recovery of estradiol was 39%. The amount of ethanol was equal to the quantity used in the experiments in which this solvent alone was employed.

When the hormone concentration was increased to 0.1, the yield of estrone fell to 1%. Changing the hormone concentration to 0.006 increased both the destruction of estradiol and the yield of estrone (Table 2). In one experiment of this series, less than 1% of the steroid was recovered (cf. Pearlman and DeMeio, 1949). These data on the effect of

TABLE 3. AEROBIC CONVERSION OF ESTRADIOL* TO ESTRONE BY DISEASED RAT LIVER

Pathology	Hormone concentration $\times 10^{-2}$	Total recovery %	Estradiol recovered		Estrone yield		
			µg	K	µg	%	K
Cirrhosis	0.02	74	735	2.0	13.1	1.3	0.33
	0.02	64	605	2.0	40.5	4.0	0.35
Hepatoma	0.02	80	805	2.1	5.9	0.6	0.33
	0.02	41	407	2.0	10.3	1.0	0.31
	0.02	70	710	2.3	6.8	0.7	0.35
Cirrhosis	0.006	60	470	1.8	138	14	0.33
	0.006	16	106	2.1	60	5.9	0.36
	0.006	1.5	0.8	2.0	0.7	0.07	0.42
Hepatoma	0.006	7.0	4.0	2.2	3.0	0.3	0.34

*—Initial estradiol = 1 mg.

TABLE 4. ANAEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY RAT LIVER SLICES

Solvent	Initial estradiol µg	Total recovery %	Estradiol recovered		Estrone yield		
			µg	K	µg	%	K
Aqueous	490	96	440	2.1	31.9	6.5	0.36
Ethanol	540	72	376	2.1	13.2	2.5	0.38

changing hormone concentration are based on a small number of experiments in which there is a great deal of variation; nevertheless, a trend is apparent.

Incubation of Estradiol with Cirrhotic Livers and Hepatoma Nodules (Table 3): When estradiol was incubated with slices of cirrhotic liver or with hepatoma nodules using a hormone concentration of 0.02 mg. hormone/mg. liver/ml. buffer the yield of estrone was much lower and the recovery of estradiol was somewhat higher than with normal tissues. When the hormone concentration was decreased to 0.006, the difference between normal, cirrhotic and malignant tissues become less marked. The variations observed in the cirrhotic livers may be attributed to differences in the severity of the disease.

Anaerobic Experiments (Table 4): When estradiol in aqueous solution was incubated in evacuated Thunberg flasks, there was some destruction of the estradiol and conversion to estrone.

TABLE 5. CONVERSION OF ESTRONE TO ESTRADIOL BY NORMAL RAT LIVER SLICES

Solvent	Gas phase	Initial estrone μg	Total recovery %	Estrone recovered		Estradiol yield		
				μg	K	μg	%	K
Ethanol	air	990	60	585	0.37	14.1	1.4	2.1
	air	990	55	530	0.37	13.8	1.4	2.3
Aqueous	air	900	45	620	0.38	2.0	0.2	2.1
Aqueous with added Ethanol	air	950	52	480	0.38	10.0	1.1	2.1
Ethanol	N_2	495	82	380	0.35	24.5	5.0	2.2

Incubation of Estrone: When estrone was incubated with rat liver slices in air or nitrogen, conversion to estradiol occurred by the yield depended on whether an aqueous or ethanol solution was used (Table 5). An experiment employing an aqueous solution of estrone with added ethanol gave results similar to those found with alcohol alone. These findings are similar to those obtained with estradiol under these varying conditions (Table 1).

DISCUSSION

Methods: In an effort to obtain quantitative data and study subtle changes, counter-current distribution and fluorometry have been employed in the present work. It has been possible by these methods to characterize as little as 1 microgram of estrogen as either estradiol or estrone and to reproduce partition coefficient within standard diviations of ± 0.02 for estrone and ± 0.1 for estradiol for hormones extracted from incubation mixtures (Table 6). From the distribution data, it is possible to calculate the amounts and purities of the components present and in addition characterize them by their partition coefficients (Engel, Slaunwhite, Carter and Nathanson, 1950).

Interconversion of Estradiol and Estrone: The data presented in Tables 1 and 5 demonstrate the interconversion of estradiol and estrone. Under the

TABLE 6. REPRODUCIBILITY OF PARTITION COEFFICIENTS BY COUNTER-CURRENT DISTRIBUTION—24 TRANSFERS IN 50% MeOH/CCl_4

Compound recovered	Number of distributions	Partition coefficient	Standard deviation	Extracted from	Reference
Estrone	85	0.36	± 0.02	Incubated tissue	Present study
	29	0.34	± 0.02	Urine	(Slaunwhite)
	—	0.33	—	Pure compound	(Engel, 1950)
Estradiol	80	2.1	± 0.1	Incubated tissue	Present study
	22	2.1	± 0.1	Urine	(Slaunwhite)
	—	2.1	—	Pure compound	(Engel, 1950)

conditions employed, the reaction appears to favor the accumulation of the later compound. The nature of the solvent and the relative amount of substrate significantly affect this reaction (Tables 1 and 2).

The relative insolubility of estrone and estradiol in aqueous media has necessitated using organic solvents to disperse the hormones in the incubation mixture. It is possible that ethanol has an inhibitory effect on oxidative enzymatic reactions. Such an inhibition seems borne out by the comparison of results of incubation of estradiol and estrone with and without ethanol. Alcohol may inhibit the oxidative breakdown of estrone, allowing its accumulation without affecting the estrone-estradiol interconversion. The latter reaction is probably catalyzed by a dehydrogenase system (DeMeio *et al.*, 1948; Coppedge *et al.*, 1948), which is unaffected by ethanol in the concentrations used. Comparable total recoveries in the aqueous and alcohol experiments make solubility differences between the two solvents less likely as a factor.

The hormone concentration also appears to be a critical factor not only in the interconversion of estradiol and estrone, but in the further changes of these compounds. The apparent discrepancies in extents of estrogen "inactivation" (Heller, 1944) which have been reported by investigators using different hormone concentrations may be explained by the dependence of the reactions on hormone concentration. If a dehydrogenase system is involved in estrogen metabolism as has been suggested by DeMeio *et al.* (1948) and Coppedge *et al.* (1948) then the critical factor related to the amount of tissue present may be the associated concentration of diphosphopyridine nucleotide. This hypothesis is supported by the differential increase of estrone yield over estradiol recovery as the hormone concentration is decreased. In other words, as the hormone concentration is decreased by increasing the amount of tissue present, there is a differential increase of dehydrogenase over oxidative activity.

Although the conversion of estrone to estradiol has been demonstrated *in vivo* (Pincus and Pearlman, 1943), the *in vitro* data have not been conclusive. Heller (1940) "activated" estrone by incubation with rat uterus slices and with cyanide inhibited liver slices. He suggested that the increased biological activity was due to the conversion of estrone to estradiol. Schiller (1945) using partitioning methods, demonstrated biological activity in the non-ketonic fraction of the "weak" phenols after perfusion of estrone through rat liver. He considered this material to be estradiol. The data in Table 5 demonstrate the conversion of estrone to estradiol by rat liver.

No attempt has been made in the present study to investigate the question of conjugation of estrogens by the liver but past work (Heller, 1940; DeMeio *et al.*, 1948; Schiller, 1945) has indicated that hydrolysis does not

increase recovery of estrogens measured biologically. The question of *in vitro* conjugation requires further study.

The problem of the site of conversion of estradiol or estrone to estriol has not been settled. Schiller (1945) reported the recovery of estrogenic activity in the "strong" phenolic fraction after perfusion of rat liver with estrone and estradiol but this observation has not been confirmed.

Metabolism of Estrogens by Cirrhotic Liver and Hepatoma: Cirrhotic liver and hepatoma nodules retain the capacity to metabolize estradiol to estrone. Microscopic sections of these tissues demonstrated them to be typical histologically, hence contamination with normal liver cannot be invoked to explain metabolic activity. It is apparent that these diseased tissues retain enzyme systems capable of oxidizing estradiol, albeit to a lesser degree than normal tissues. When the hormone concentration was decreased sufficiently (0.006), the differences between normal and diseased tissues diminished. Again it should be noted that these conclusions are based on a small series in which there was a high degree of variability. In one experiment, hepatoma did not effect the conversion of estrone to estradiol.

Anaerobic Experiments: When estradiol or estrone was incubated with rat liver slices in evacuated Thunberg flasks or in an atmosphere of nitrogen, interconversion was observed but the recovery of added steroid was much higher than in the aerobic experiments. This observation is in disagreement with DeMeio *et al.* (1948) who reported no "inactivation" in nitrogen. Actually, his data revealed 9 to 17% inactivation but this was, as he stated, within the limits of error of the bioassay method. On the other hand, Levy (1947) reported 30–50% inactivation under anaerobic conditions. The finding of metabolic change in nitrogen does not, however, discredit the theory of a DPN linked reaction since a coupled oxidation-reduction system could provide enough hydrogen acceptors to reoxidize the reduced DPN and change limited amounts of estradiol or estrone in the absence of oxygen. By analogy with anaerobic glycolysis, a reduction would tend to be favored under anaerobic conditions.

SUMMARY

1. Counter-current distribution and photofluorometry have been applied to the separation, characterization and quantitative estimation of estrone and estradiol after incubation with rat liver slices.

2. The interconversion of estrone and estradiol occurs under both aerobic and anaerobic conditions.

3. Ethanol has a slight inhibitory effect on the oxidative breakdown of estrone and estradiol.

4. The extent of conversion of estradiol to estrone by normal and cir-

rhotic rat livers and hepatoma nodules depends on the hormone concentration.

Acknowledgements

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p. 288. Paragraph 1, line 4 should read " . . . in the incubation were 0.0001 and 0.0002 mg. of hormone . . . ."

Table 1 Column 4. Heading should read, "Estradiol Concentration* $\times 10^2$."

Table 2 Column 4. Heading should read, "Estrone Concentration* $\times 10^2$."

Table 3 Column 4. Heading should read, "Estriol Concentration* $\times 10^2$."

THE INTERCONVERSION OF ESTRONE AND ESTRADIOL BY HUMAN TISSUE SLICES[1,2,3]

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STUDIES of estrogen metabolism in animals have limited application to man because of the different metabolic pathways in different species. For example, estriol has been recovered only from human sources; epiestradiol is a product of rabbit and equine metabolism and the naphtholic estrogens are peculiar to equine species (Pincus and Pearlman, 1943). Comparative studies of the *in vitro* metabolism of estrogens by human and animal tissues have not been extensive, but species differences have been noted (Twombly and Taylor, 1942; Szego and Samuels, 1943; Samuels and McCauley, 1946). The biochemical interrelationships between steroid hormones and cellular function is at present a matter of intense interest. In order to measure directly the metabolic activity of normal and neoplastic tissues of man, an investigation of estrogen metabolism in such tissues was undertaken. The data revealed that the interconversion of estrone and estradiol as well as the further degradation of both of these compounds can be accomplished by a variety of normal and diseased human tissues.

A previous report (Ryan and Engel, 1953) described the application of counter-current distribution and photofluorometry to the separation, characterization and quantitative estimation of estrogens after incubation with rat liver slices. These same procedures have now been applied to the study of the metabolism of estrone, estradiol and estriol by human tissues.

METHODS

Tissue Preparation: All tissues were operative specimens which were obtained under aseptic conditions and kept on ice for a few minutes until ready for incubation. Slices

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[2] A preliminary report of this work was presented at the Second International Congress of Biochemistry, Paris, France, July 21–26, 1952.

[3] This is publication #778 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

were cut on a Stadie-Riggs microtome to 0.5 mm. thickness, blotted dry with filter paper and weighed. Sections were made for histologic diagnosis. In all cases, control specimens of tissue were extracted and analyzed fluorometrically. No contaminating estrogens were found.

Hormone Preparation: The specifications of the estrone and estradiol, the buffer medium and the techniques of incubation, extraction and analysis have been described (Ryan and Engel, 1952). Ethanol solutions of the hormones were used throughout and the concentrations of the steroids in the incubations were 0.01 and 0.02 mg. of hormone /mg. tissue/ml. buffer. The amounts of hormone ranged from 450–1020 μg.

Estriol[5] was freed from a small amount of contaminating estrone (1.2%) by an eight transfer counter-current distribution in 50% MeOH/CCl_4. The stock solution was prepared in absolute ethanol at a concentration of 5 mg./ml.

TABLE 1. CONVERSION OF ESTRADIOL TO ESTRONE BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estradiol concentration*	% Estradiol recovered	% Estrone yield
Placenta	Term	Air	.01	33	38
Placenta	4 month	Air	.01	31	11
Ileum	Normal	Air	.01	35	11
Liver	Cholangitis	Air	.02	62	5.9
Liver	Cholangitis	Air	.02	51	4.6
Liver	Cholangitis	N_2	.02	60	2.1
Adrenal	Hyperplastic	Air	.02	56	2.3
Adrenal	Hyperplastic	N_2	.02	55	0.56
Adrenal	Cushing's	Air	.02	57	1.0
Breast	Pregnancy	Air	.01	43	2.3
Breast	Pregnancy	N_2	.01	61	3.1
Breast	Fibroadenoma	Air	.01	63	1.3
Breast	Fibroadenoma	N_2	.01	82	0.56
Breast	Adenocarcinoma	Air	.02	50	0.60
Endometrium	Hyperplastic	Air	.02	75	1.7
Endometrium	Hyperplastic	N_2	.02	61	0.92
Myometrium	Fibroid	Air	.02	54	0.66
Myometrium	Fibroid	N_2	.02	61	0.45
Testes	Normal	Air	.02	71	0.50

* Hormone concentration = mg. Estradiol/mg. tissue/ml. buffer.

RESULTS

The results are presented in Tables 1 to 3. All tissues studied were found to have the ability to metabolize estrogens, but there are differences in the types and extents of the reactions. Total recoveries varied from 42 to 92% depending on the hormone used, the tissue with which it was incubated and the gas phase under which the experiment was conducted. Metabolic activity was observed under anaerobic conditions. Similar findings have been reported for rat liver (Ryan and Engel, 1953). Tissues of the digestive, reproductive and endocrine systems of man can convert estradiol to estrone. The 18–58% of the hormones unaccounted for represents conversion to as yet unknown metabolites, in addition to loss

[5] We wish to thank the Schering Corporation and Parke, Davis and Company for the gifts of the estriol samples.

during extraction and analysis. This latter loss has been found to be approximately 10% of the starting material (Ryan and Engel, 1953).

Estriol was recovered almost quantitatively after incubation with testis and term placenta. Four month placenta gave a lower recovery of estriol (Table 3). In none of these three experiments was estrone or estradiol found after incubation of estriol.

TABLE 2. CONVERSION OF ESTRONE TO ESTRADIOL BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estrone concentration*	% Estrone recovered	% Estradiol yield
Testes	Normal	Air	.02	47	7.0
Placenta	4 month	Air	.01	64	1.4
Placenta	Term	Air	.01	59	0
Kidney	Hydronephrosis	Air	.02	65	0.69
Endometrium	Hyperplastic	Air	.02	62	0.51
Adrenal	Hyperplastic	Air	.02	43	0.46
Adrenal	Hyperplastic	N_2	.02	62	0
Breast	Pregnancy	Air	.01	72	0.34
Myometrium	Fibroid	Air	.02	80	0

* Hormone concentration = mg. Estrone/mg. tissue/ml. buffer.

TABLE 3. INCUBATION OF ESTRIOL WITH HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estriol concentration*	% Estriol recovered
Testes	Normal	Air	.02	92
Placenta	Term	Air	.01	92
Placenta	4 month	Air	.01	67

* Hormone concentration = mg. Estriol/mg. tissue/ml. buffer.

DISCUSSION

Except in the cases of the testes, term placenta and ileum, the tissues used were abnormal histologically and/or functionally, therefore, the estrogen metabolism of these tissues is not necessarily comparable with their normal counterparts.

Normal Tissue: The testis, in marked contrast to the other tissues studied, converts estrone to estradiol to a greater extent than the reverse reaction. A possible explanation for this phenomenon is that the enzyme systems ordinarily involved in testosterone synthesis from Δ^4-androstene-3,17-dione are accomplishing the estrone-estradiol conversion. Dorfman (1948), Clark and Kochakian (1947), and Kochakian *et al.* (1952) have shown an intimate relation between androstenedione and testosterone and suggested that androstenedione may be a precursor of testosterone. The reaction concerned is the reduction of the 17-ketone group of androstenedione to the 17β-hydroxyl group of testosterone. It is possible that the 17-ketone

group of estrone is reduced to the 17-hydroxyl group of estradiol by the same enzyme system.

One incubation of estradiol with terminal ileum resulted in an 11% yield of estrone (Table 1). This yield is higher than that found with any tissue other than placenta.

Placental tissue has by far the greatest activity of all the tissues studied, 11 and 38% yields of estrone being realized with four month and term placental tissue respectively. However, the reduction of estrone to estradiol by these tissues is minimal (Tables 1 and 2).

Abnormal Tissues: In confirmation of the work of Twombly and Taylor (1942), Samuels and McCauley (1946) and more recently Tagnon *et al.* (1952), the degradation of estradiol by human liver slices has been demonstrated. In addition, it has been shown here that this tissue can oxidize estradiol to estrone as well as degrade both to unrecognizable metabolites (Table 1).

Endocrine System: Adrenal cortex slices can effect the interconversion of estrone and estradiol (Tables 1 and 2). This is not surprising in view of the intense activity of this tissue in steroidogenesis.

Slices of human pregnancy breast, mammary fibroadenoma and adenocarcinoma (grade iii) can convert estradiol to estrone with comparable facility. Hyperplastic endometrium and uterine fibroid tissues have a similar activity. This is in contrast to the findings of Szego and Samuels (1943) who reported no conversion of estrogens by endometrial tissue taken from a woman in the third month of pregnancy. In one experiment, kidney tissue converted estrone to estradiol.

Under the experimental conditions employed, estriol does not seem to be a significant metabolic conversion product of either estrone or estradiol in any of the tissues studied. Moreover, testis and term placenta do not appear to have the capacity to cause destruction of estriol; hence, if it were formed by these tissues in any significant quantity it should have been detectable. The techniques used permit the detection and characterization of a minimum of 5 μg. of estriol.

The demonstration that a wide variety of human tissues can metabolize estrogens requires that the concept of the liver as the only and/or chief site of estrogen metabolism be questioned. It appears that such organs as breast, uterus, small bowel, testis, adrenal cortex, kidney and placenta are capable of metabolizing significant amounts of estrone and estradiol, and thus must be included in any consideration of the over-all metabolism of these compounds.

SUMMARY

1. A variety of normal and abnormal human tissues have the capacity to

interconvert estrone and estradiol and to degrade both to unrecognizable metabolites.

2. No conversion of estrone or estradiol to estriol was observed.

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INHIBITION OF DEVELOPMENT OF DCA HYPERTENSION BY A NEW SYNTHETIC STEROID

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IT HAS been well established that desoxycorticosterone acetate (DCA) administered to normal rats or to rats sensitized by high sodium intake and/or uni-nephrectomy will produce renal and vascular pathology accompanied by arterial hypertension (Selye, Hall, and Rowley, 1943; Friedman, Polley, and Friedman, 1948; Masson, Corcoran, and Page, 1949). Although the etiology of DCA hypertension has not been explained in a manner satisfactory to all workers in the field, there have been searches for agents which will either reverse an established DCA hypertension or prevent the development of DCA hypertension in experimental animals, both because of the possible clinical implications and because the use of such agents would be helpful in research on mechanisms in vascular disease.

Agents which have been reported to be effective in preventing the development of DCA hypertension in rats are: *Lipo-Adrenal Cortex*,[1] estradiol[2] (Friedman and Friedman, 1950), cortisone acetate[2] (Friedman, Friedman, and Nakashima, 1950), and Phenergan (Guillemin and Fortier, 1951).

Agents which have been without inhibitory effect on development of DCA hypertension are Compound S acetate (Masson, Corcoran, and Page, 1950), progesterone, testosterone, hyaluronidase, "acetoxy-pregnenolene," "saturated 12-keto-DCA" (Friedman and Friedman, 1950), and methylandrostenediol (Skelton, 1952).

The present paper presents data on a new steroid, "U-0160," 3,21-diacetoxy-5,7,9, pregnatrien-20-one maleic anhydride adduct (Levin,

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[1] Registered Trade-Mark, The Upjohn Company, for cottonseed oil solution of hog adrenal extract.

[2] The authors cited do not feel that the anti-hypertensive action of estradiol and of cortisone acetate represent true antagonism to the "cardiovascular-renal effects of DCA" because of the debilitation of the animals receiving them. Cortisone, in their experiments, did not prevent the cardiac and renal enlargement produced by DCA. It did, however, partially prevent the DCA-induced rise in blood pressure. The effect of estradiol was termed "non-specific," although it alleviated the hypertensive effect of DCA very markedly.

Spero, McIntosh, Wesner, Meinzer, Searcy, and Thompson, 1951) which has the ability to prevent the blood pressure changes which accompany administration of DCA to a sodium-sensitized, uni-nephrectomized rat. The structure of this compound is shown in Figure 1.

METHODS AND MATERIALS

A. *Animals.* All experments were carried out on young male rats of the Upjohn strain (a colony developed from Sprague-Dawley stock), 120 to 140 grams body weight

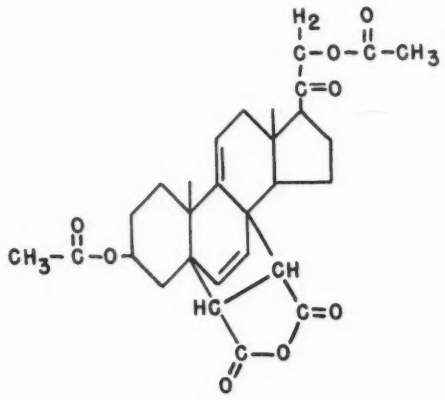

FIG. 1. Structural formula of U-0160.

at the beginning of the experiment. The rats were unilaterally nephrectomized (left kidney) and were given a diet of Archer dog food and 1% sodium chloride drink. Beginning on the day following nephrectomy and continuing until the end of the experiment, all animals were injected with (A) 2 mg DCA, (B) 2 mg DCA plus 2 mg U-0160, (C) 2 or 4 mg U-0160, or (D) cottonseed oil only. All preparations were made so that the daily dose was contained in 0.2 ml cottonseed oil. This concentration exceeds the solubility of U-0160, and the resulting product was a finely-divided suspension.

All animals were injected once daily for 16 or 17 days. All rats' blood pressures were estimated three times during the experiments: The first two sets of determinations were taken to accustom the animals to the apparatus (see below for description of blood pressure estimation technique), and to trace the course of blood pressure changes. The definitive values, however, are those obtained on the seventeenth to eighteenth days, the day before sacrifice of the rats.

The rats were killed with chloroform and autopsied on the day following the last blood pressure estimation. The whole animal, the cardiac ventricles, the kidney, and, in some animals, the adrenal and thymus glands were weighed. Careful attention was given to the presence or absence of gross lung lesions, as it has been found that the occurrence of infected lungs is associated with highly aberrant blood pressures.

All experiments were designed with five or six animals in a single dosage group. Due to the occurrence of lung lesions in a number of animals and the necessity for discarding

the contributions of these animals to the data, the results reported below include a number of groups of three or four rats.

Essentially the same experiment was performed at three widely separated times, as described with the results presented below.

B. *Blood Pressure Estimations.* The blood pressure estimations were carried out on conscious, unrestrained rats by a method involving measurement of optical density of the right hind foot during the deflation of a pneumatic cuff wrapped around the lower leg. The apparatus was constructed in The Upjohn Company Department of Physics by Mr. C. D. Alway and Mr. D. C. Cummings, incorporating principles described by two other laboratories: Kersten, Brosine, Ablondi, and SubbaRow (1947) found that a characteristic change in light transmittance of the rat foot occurred when the descending

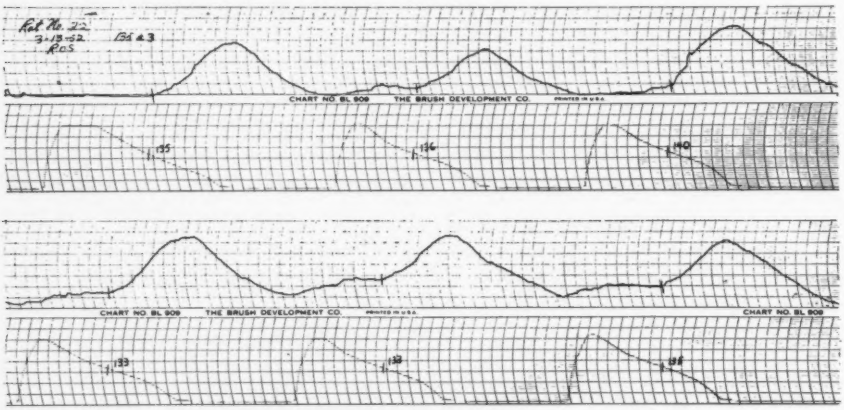

FIG. 2. Photograph of a record of blood pressure estimation with the Upjohn photoelectric tensometer. The record has been cut in half for conservation of space. The lower line indicates air pressure in the cuff, the upper line indicates photocell current. The point at which the upper line changes direction sharply is indicative of blood flow into the foot.

external pressure equalled the systolic blood pressure; Olmsted, Corcoran, Glasser, and Page (1948) described an apparatus which provided simultaneous recordings of the cuff pressure and the volume of the foot measured with a strain gauge.

Our apparatus is constructed so that an intermittent light source of a specific frequency is transmitted through the foot of the lightly-held rat and impinges upon a photoelectric cell. The photo-cell current, routed through a series of amplifiers, activates the pen of a Brush Development Co. recorder. The pneumatic pressure in the bulb-cuff system is reflected by the expansion and contraction of a bellows which, through a rack-and-pinion mechanism, activates a potentiometer connected with a second pen on the Brush recorder.

The blood pressure work was all carried out in an insulated "quiet room" with extraneous noise reduced to a low level. The rats were handled as gently as possible and were placed in the rat holder in a manner designed for comfort and freedom from excessive restraint. After the first few determinations the animals were generally cooperative.

The variation in results obtained by the method is fairly large. We, therefore, rou-

TABLE 1. BLOOD PRESSURE ESTIMATIONS

Date	Treatment[2]	No. rats	Systolic blood pressures in mm Hg.[1]		
			I	II	III
4-51			*Days 5 and 6*	*Days 11 and 12*	*Day 18*
	Control[3]	6	129 (114–144)	129 (120–139)	136 (128–144)
	DCA	6	142 (118–166)	156 (138–184)	173 (153–193)
	DCA plus U-0160	6	119 (102–137)	122 (99–151)	140 (135–150)
	U-0160	6	138 (128–157)	128 (115–140)	125 (102–153)
6–51			*Days 10 and 11*	*Day 14*	*Day 17*
	Control[3]	4	127 (118–138)	133 (119–150)	130 (126–135)
	DCA	5	160 (135–202)	167 (140–197)	171 (146–197)
	DCA plus 2 mg. U-0160	5	130 (105–154)	122(110–135)	129 (115–135)
	DCA plus 4 mg. U-0160	4	126 (109–160)	130 (122–143)	133 (121–146)
	U-0160[4]	5	147 (126–190)	128 (119–140)	140 (128–169)
3-52			*Days 2 and 3*	*Day 8*	*Days 17 and 18*
	Control[3]	3	118 (101–141)	137 (123–164)	133 (127–138)
	DCA	5	112 (100–125)	154 (133–169)	167 (152–186)
	DCA plus U-0160	4	114 (98–136)	113 (105–123)	129 (120–142)
	U-0160	5	129 (120–135)	118 (94–141)	120 (101–151)

[1] Figures in parentheses indicate extremes within groups. Days indicated are days after nephrectomy.
[2] All doses 2 mg. per rat per day unless otherwise indicated.
[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.
[4] Suspended in saline. All other preparations suspended in cottonseed oil.

TABLE 2. BODY AND ORGAN WEIGHTS[1]

Date	Treatment[2]	No. rats	Body weights	Cardiac weights	Kidney weights
4-51	Control[3]	6	179 (155–214)	324 (308–367)	705 (594– 968)
	DCA	6	160 (125–182)	442 (336–457)	823 (700– 920)
	DCA plus U-0160	6	174 (156–201)	380 (358–408)	779 (708– 860)
	U-0160	6	175 (155–218)	327 (319–324)	634 (542– 784)
6-51	Control[3]	4	171 (157–182)	308 (290–340)	640 (590– 690)
	DCA	5	187 (172–203)	379 (350–440)	882 (740–1100)
	DCA plus 2 mg. U-0160	5	131 (110–156)	336 (310–400)	974 (910–1060)
	DCA plus 4 mg. U-0160	4	151 (139–163)	355 (330–380)	1237 (1070–1380)
	U-0160[4]	5	175 (166–188)	340 (310–370)	738 (660– 910)
3-52	Control[3]	3	199 (174–220)	304 (293–320)	723 (690– 825)
	DCA	5	169 (150–190)	366 (350–405)	902 (800–1020)
	DCA plus U-0160	4	152 (128–188)	292 (202–352)	1044 (755–1185)
	U-0160	5	176 (166–188)	287 (271–298)	885 (668–1397)

[1] Organ weights expressed as mg. per 100 g. body weight. Figures in parentheses indicate extremes within groups.
[2] All doses 2 mg. per rat per day unless otherwise indicated.
[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.
[4] Suspended in saline. All other preparations suspended or dissolved in cottonseed oil.

tinely obtained five to nine tracings on each rat and used the mean value as the representative systolic blood pressure. The source of the variation is chiefly in the spontaneous changes in position of the rats' feet (and possibly emotion-induced blood pressure changes) during the estimation period, since variation obtained with anesthetized animals is minimal.

Figure 2 is a photograph of a recording of a single blood pressure estimation on one rat. The scale of the recording paper is calibrated so that pressure is read in millimeters of mercury.

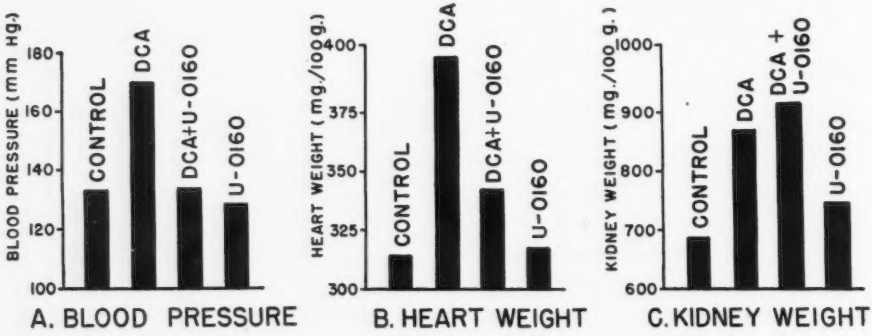

FIG. 3. Pooled data from three experiments, showing mean blood pressure, heart weight, and kidney weight of all rats at the end of the experiment. A. Terminal blood pressure; B. Weight of cardiac ventricles; C. Weight of kidney.

RESULTS AND DISCUSSION

The data on blood pressures are presented in Table 1, autopsy data in Table 2. The terminal data on all three experiments are summarized graphically in Figure 3.

The control animals (nephrectomy, high sodium intake, blank injections) showed a rise in blood pressure during the course of the experiments, which is considered to be a mild hypertension. This pressure rise is commonly observed in this type of animal under these conditions.

In the rats receiving only DCA, we observed a consistent development of hypertension, accompanied by renal and cardiac weight increase. When U-0160 was given to animals receiving DCA, however, there was complete inhibition of the hypertension and substantial inhibition of the cardiac hypertrophy, but no inhibition of the increase in renal mass. When one considers mean values from three experiments, there was augmentation of the renomegalic effect of DCA when U-0160 was given.

The administration of U-0160 alone in two experiments resulted in blood pressures lower than those of the control animals. The compound by itself had no effect on relative heart weight, but in two of the three experiments induced an increase in kidney mass.

In attempting to explain the action of U-0160 on the blood pressure of the DCA-treated rat, one must consider the hypothesis that the compound is producing a "non-specific stress" and that the inhibition of DCA hypertension is caused by an elevation in secretion of 11-oxygenated steroids from the adrenal cortices. This is particularly attractive because cortisone (Friedman, Friedman, and Nakashima, 1950) and *Lipo-Adrenal Cortex* (Friedman and Friedman, 1950) antagonize the hypertensive action of DCA. We have found (unpublished data) that corticosterone and hydrocortisone also possess this property.

It is doubtful that the action of U-0160 is through such a mechanism, however, because there is no evidence of adrenal hypertrophy or thymic

TABLE 3. ADRENAL AND THYMUS WEIGHTS OF CONTROL, DCA-TREATED, AND U-0160-TREATED RATS

Experiment	Treatment	No. rats	Adrenal weights		Thymus weights	
			Absolute	Mg./100 gm.	Absolute	Mg./100 gm.
4-51	Controls	6	41.2	23.7	330	190
	DCA	6	36.7	23.6	335	208
	DCA plus U-0160	6	40.1	23.3	328	188
	U-0160	6	40.2	23.3	350	199
3-52	Controls	3	45.9	23.3	—	—
	DCA	5	36.7	22.1	—	—
	DCA plus U-0160	4	37.2	25.1	—	—
	U-0160	5	39.3	22.4	—	—

atrophy in the U-0160-treated rats. Table 3 shows the mean adrenal weights obtained in two experiments and the thymic weights in one experiment; far from showing hypertrophy, the adrenals of the animals receiving U-0160 are slightly smaller than those of the control animals in both instances. There is no significant change in thymus weight. These findings are not characteristic of a "stressed" animal.

One might speculate that the compound is functioning as an "antimetabolite" with respect to DCA in a manner analogous to the relation between folic acid and its antagonists, i.e., being structurally related so as to form undissociable complexes with cellular enzymes, blocking the effect of the DCA on cellular physiology in this manner. The presence of the non-physiologic groups on the molecule leads one to suspect such a mechanism. Preliminary evidence indicates that the maleic anhydride group is necessary for the pharmacological action of the compound, although maleic anhydride *per se* is without effect on DCA hypertension. These findings are summarized in Figure 4. U-0160 minus the maleic anhydride group (3,21 diacetoxy-5,7,9-pregnatrien-20-one) is designated in our laboratories as "U-0176."

Note that administration of U-0176 did not antagonize the effects of DCA on blood pressure to any significant extent, nor did the administration of 2 mg per day of maleic anhydride.

The increase in renal mass caused by U-0160 is interesting, and may play a role in the blood pressure effects produced by this compound. We have insufficient data to attempt to explain the renal effects of U-0160 because the kidneys of these animals are undergoing changes which are the resultant of several factors: compensatory hypertrophy as a result of removal of the contralateral kidney, response to the sodium-DCA-induced

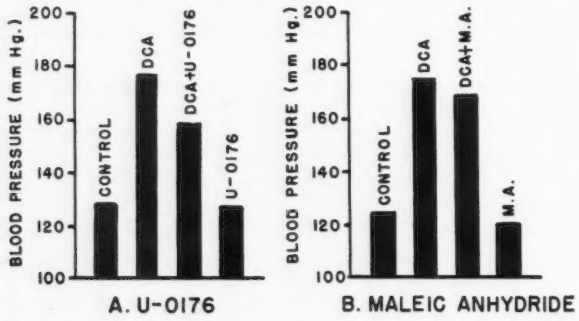

FIG. 4. A. Terminal blood pressures (Day 17–19) of rats receiving 2 mg DCA per day and/or 2 mg U-0176 per day; pooled data of two experiments similar in design to those shown in Figure 2. B. Terminal blood pressures (Day 19) of rats receiving 2 mg DCA per day and/or 2 mg maleic anhydride in aqueous solution. Single experiment, four or five rats per dose.

diuresis, and response to the renomegalic effect of DCA itself. The role of U-0160 in the structure and physiology of this type of kindey is, therefore, a research problem in itself.

SUMMARY

U-0160 (3,21 diacetoxy-5,7,9-pregnatrien-20-one maleic anhydride adduct) was effective in preventing the hypertension produced by DCA in the unilaterally nephrectomized, sodium-sensitized rat. This property is not shared by U-0176 (U-0160 less maleic anhydride) or by maleic anhydride itself.

U-0160 also largely prevents the cardiac hypertrophy accompanying DCA hypertension, but augments the increase in renal mass.

It is considered improbable that the action of the compound is through release of 11-oxygenated hormones from the adrenal cortex, as there is no evidence of adrenal hypertrophy or thymic involution produced by the compound.

Acknowledgments

The authors are grateful to Dr. R. H. Levin, The Upjohn Company, Kalamazoo, Michigan, for generous supplies of U-0160 and U-0176 and for his criticism of this manuscript.

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ASSAY OF ACTH ACTIVITY BASED ON THE EOSINOPENIC RESPONSE IN NORMAL AND HYPOPHYSECTOMIZED MICE[1]

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SINCE 1937, a number of assay procedures for ACTH have been proposed. These have generally been based upon a change in size of the adrenal glands of normal rats and baby chicks, or changes in the adrenal glands of hypophysectomized animals. The hypophysectomized rat has been the animal of choice, and the effect of ACTH on adrenal repair, maintenance, ascorbic acid or cholesterol levels have been used as end points in the assay. The ascorbic acid depletion test proposed by Sayers, *et al.* (1948) has proved to be the most satisfactory one. It is an extremely sensitive test, reasonably accurate and very useful in detecting small amounts of hormone in body fluids. (Gemzell, 1952.)

At the present time, the role of ascorbic acid in the synthesis or release of adrenal cortical hormones is unknown (Sayers, 1950). The factor in the ACTH that brings about the change in ascorbic acid level, may not be the same factor that produces an increase in size of the adrenal or release of the adrenal hormones. Reinhardt, *et al.* (1951a, b and c) compared the results of the ascorbic acid depletion test with those of the eosinopenia and adrenal maintenance tests. He found that the results do not compare favorably. A high ascorbic acid depleting power does not necessarily correspond to a high rate of secretion of the cortical hormones as measured by a reduction in eosinophils or in thymus and lymph node weights. These data have led a number of workers to suggest that in order to obtain accurate results in the assay of ACTH preparations several bioassay methods must be used (Greenspan, Li, Simpson and Evans, 1950; Selye, 1951).

In recent years a bioassay method for adrenal cortical hormones has been developed which is extremely sensitive, accurate and relatively easy to perform in mice (Speirs and Meyer, 1951; Speirs, Wragg, Bonner and Homburger, 1951). This assay is based upon the ability of the adrenal

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cortical hormones to reduce the number of circulating eosinophils in adrenalectomized mice during a three hour period. It should be possible to modify this eosinophil method to assay ACTH. The assay would then measure the ability of ACTH to release the 11-oxycorticosteroid hormone from the adrenal cortex and would thus differ from the ascorbic acid depletion, repair or maintenance tests, and could be used to supplement them. Furthermore, it would be possible to measure the duration of the ACTH action upon the adrenal releasing the hormone. Such an assay might be very useful in developing and standardizing long-acting ACTH preparations.

The following paper is a report of a series of investigations to determine if the eosinopenic response of normal mice can be used to assay long acting ACTH preparations.

MATERIAL AND METHODS

The mice used in these experiments were the BBF_1 hybrid animals developed for the assay of adrenal cortical hormones; mice of strain 129 were used for comparison. The BBF_1 mice are the offspring of a cross between C57BR/cd and C57BL/6. Only male mice approximately 6 weeks of age and weighing between 20 and 25 grams were used.

The ACTH used in these experiments was supplied by Armour and Company, and assayed by them according to the ascorbic acid procedure. These hormones were used in order to determine possible assay procedures and no effort has been made to compare the eosinopenic values of various types of ACTH.

The techniques of performing eosinophil counts described earlier (Speirs and Meyer, 1949; Speirs, 1952a) have been modified slightly. Standard WBC diluting pipettes were used and the cells were counted in an Eosinophil Counting Slide (Speirs, 1952b) using a bifocal microscope containing 15× wide field oculars and 16 mm. objective. The eosinophil diluting fluid consisted of the following:

Phloxine B	0.02	gm.
Diethylene Glycol	2	ml.
Alconox (or similar detergent)	0.002	gm.
Distilled Water	85	ml.
Acetone, added just before using	15	ml.

Precautions were taken in order to avoid an eosinopenia due to stress. To avoid disturbances due to fighting, etc., the mice were kept in individual cages which were placed in a dark cabinet maintained at 27° C. for at least 24 hours prior to the experiment. Food, consisting of Purina Fox Chow with a weekly supplement of rolled oats, and water, were given ad libitum. Eosinophil counts were performed within 10 minutes after the animals were first disturbed. They were removed from their cages and placed in a battery jar containing wood chips and warmed by a 100 watt lamp. As soon as the animals became active, their tails were washed in warm soapy water, rinsed, and a small nick made through the skin and through one of the prominent blood vessels. Profuse bleeding occurred in all cases. The first two drops of blood were discarded, and fresh blood was drawn into the WBC pipette and diluted 1:20 with the above-mentioned diluent. One pipette of blood was taken from each animal, and from that two chambers on the Eosinophil Counting Slide were loaded. After allowing the cells to settle and stain for approximately 15 minutes, the eosinophils in both chambers were counted. The total count of both chambers (twenty 1×1×0.2 mm. volumes) was multiplied by 5 to obtain the number of cells per cu. mm. of blood.

The changes in circulating eosinophils were given in this paper as percent decreases. They were computed as follows:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$$

All positive values (an increase in eosinophils) were recorded as zero percent decreases.

PROCEDURE AND RESULTS

In designing any assay procedure involving the fluctuation of eosinophils in normal animals, one must first consider the normal variation of these cells, particularly the diurnal variation (Halberg, 1951). In order

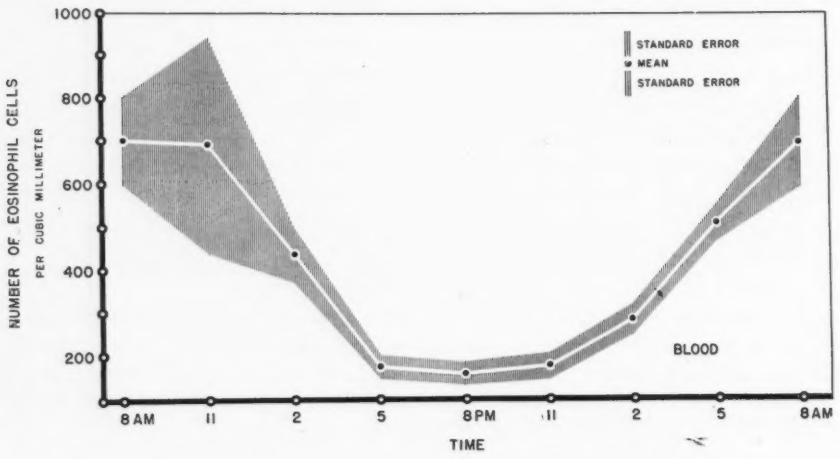

FIG. 1. Diurnal Variation of Eosinophil Cells in Blood. Each point represents the mean and standard error of 12 normal male BBF_1 mice.

to determine this in our animals, normal male mice were maintained in individual cages and kept in a dark cabinet with ample food and water. These mice were considered to be relatively undisturbed or free of stress prior to the experiment. At three hour intervals throughout the day and night, groups of animals were removed from their cages without disturbing the remaining animals, and eosinophil counts were performed. Each animal was used for only one eosinophil determination. This was repeated on two separate occasions and a total of 12 eosinophil counts were obtained for each period. The mean eosinophil counts and the standard error of the mean are plotted in Figure 1.

It may be seen that the number of eosinophils fluctuates greatly during the day. A peak of 700 cells per cu. mm. was obtained at 8 and 11 A.M. and a low of 155 cells occurred between 5 and 11 P.M.

The next factor to consider is the effect of mild stress upon the number of circulating eosinophils. Figure 2 illustrates the mean and standard error of the number of eosinophils in normal C57 Brown mice following mild stress such as handling (from Speirs and Meyer, 1949). A similar eosinopenia was also obtained in the BBF_1 mice (unpublished data). Thus an

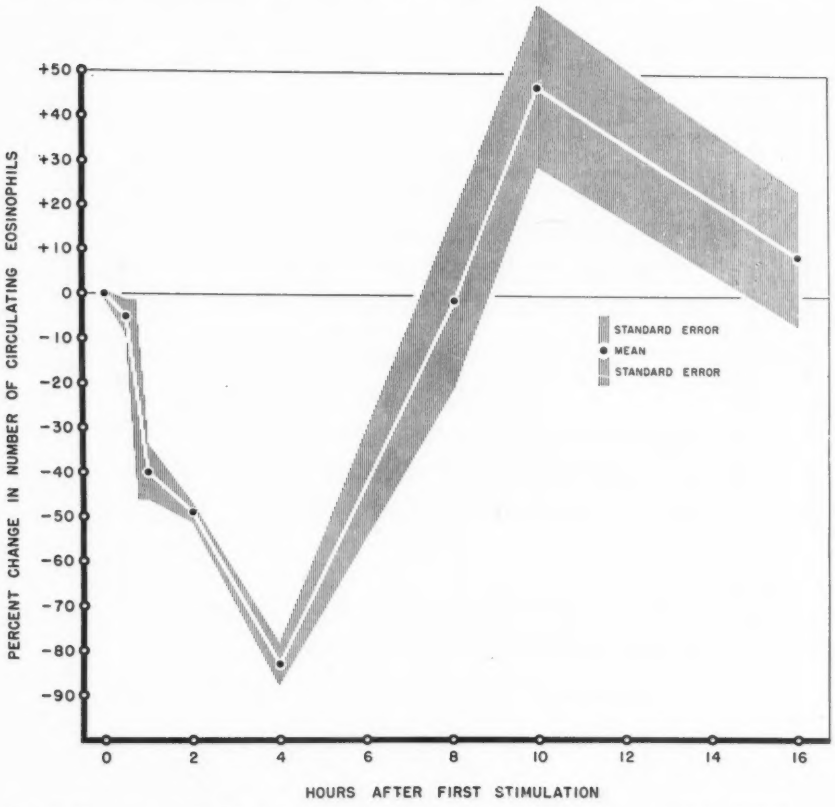

FIG. 2. Variation in the Number of Circulating Eosinophils in Normal Male C57 Brown Mice Following Mild Stress. Each point represents the mean and standard error of 9 mice.

eosinopenia occurs in these animals following mild stress. There is complete recovery within 24 hours after the animals are submitted to a single mild stress.

Figure 3A shows the response curve of mice which had received subcutaneously 2 I.U. of ACTH in $\frac{1}{4}$ cc. of saline. This curve is essentially the same as the one obtained following a mild stress. By 10 hours recovery had begun and was complete by 18 to 24 hours.

Figure 3B presents results obtained following the subcutaneous injection of 2 I.U. of ACTH suspended in 0.1 cc. of gelatine. It may be seen that in the 6 and 10 hour groups, there was a decrease in eosinophils of 98% and 99%. Recovery began by 18 hours and was complete by 24 hours.

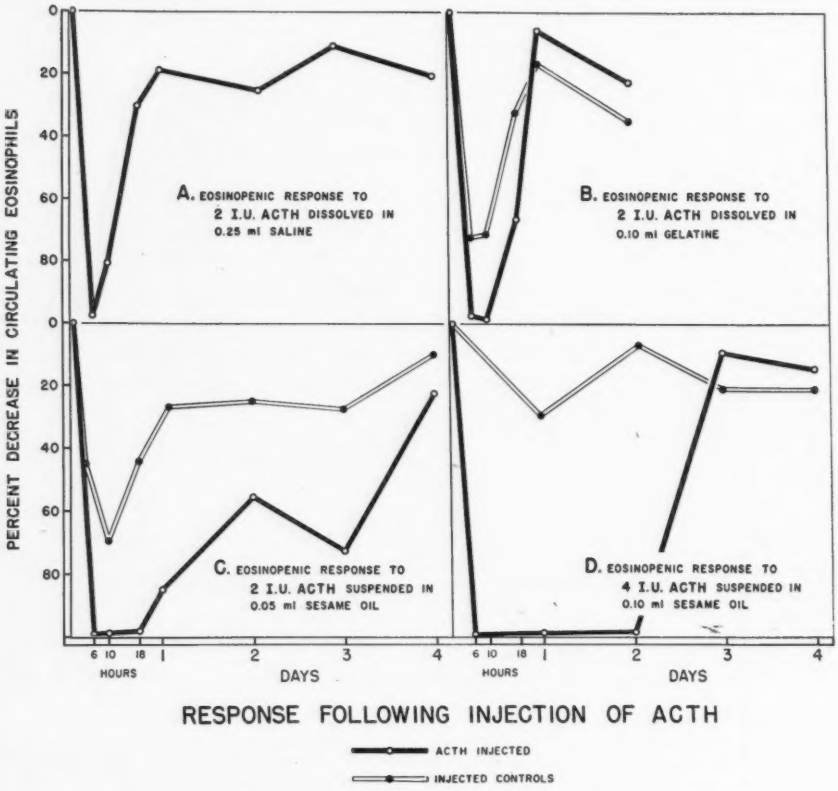
PERCENT DECREASE IN CIRCULATING EOSINOPHILS
A. EOSINOPENIC RESPONSE TO 2 I.U. ACTH DISSOLVED IN 0.25 ml SALINE
B. EOSINOPENIC RESPONSE TO 2 I.U. ACTH DISSOLVED IN 0.10 ml GELATINE
C. EOSINOPENIC RESPONSE TO 2 I.U. ACTH SUSPENDED IN 0.05 ml SESAME OIL
D. EOSINOPENIC RESPONSE TO 4 I.U. ACTH SUSPENDED IN 0.10 ml SESAME OIL
6 10 18 1 2 3 4
HOURS
DAYS
RESPONSE FOLLOWING INJECTION OF ACTH
ACTH INJECTED
INJECTED CONTROLS

FIG. 3. Eosinopenic Response of Normal Male BBF_1 Mice to ACTH. Each point represents a mean obtained from at least 6 mice.

Figures 3C and D show animals which were injected with the ACTH suspended in sesame oil. In the group receiving 2 I.U., there was a complete absence of eosinophils 6 hours after the injection, and a 99% and 98% decrease in the 10 and 18 hour groups. Twenty-four hours following the injection an eosinopenia of 85% was found. Recovery began by 48 hours. In the group receiving 4 I.U. of ACTH suspended in sesame oil recovery did not occur until 72 hours following the injection.

It should be noted that in all the animals which received control in-

jections, complete recovery in the number of circulating eosinophils occurred within 24 hours. This agrees with earlier published data in which the effects of epinephrine and mild stress were studied (Speirs and Meyer, 1949). However, the ACTH suspensions in gelatine and oil produced an eosinopenia which persisted for 24 hours or more. These data suggested

TABLE 1. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH DISSOLVED IN GELATINE

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS
			0 20 40 60 80 100
0.25 ml.	0.5	6	50 ± 11.1
	1.0	6	59 ± 6.5
	2.0	5	95 ± 2.1
	3.0	6	97 ± 1.3
	5.0	6	99 ± 0.4
0.5 ml	0.5	6	58 ± 11.8
	1.0	6	55 ± 9.5
	2.0	6	96 ± 1.0
	4.0	5	100 ± 0.4
	10.0	6	100 ± 0
0.1 ml	2.0	12	6 ± 3.1
0.25 ml	2.0	5	95 ± 2.1
0.5 ml	2.0	6	96 ± 1.0
0.25 ml	0	6	25 ± 9.3
0.5 ml	0	6	51 ± 13.6 CONTROLS

that possibly an assay for ACTH could be developed in which only the 24 hour response was considered.

Table 1 summarizes the data in a group of 76 mice which had received various gelatine suspensions of ACTH. In the first group the ACTH was suspended in 0.25 ml. of gelatine. It may be seen that 0.5 I.U. of ACTH produced an average eosinopenia of 50%, 24 hours after the injection. One I.U. produced a slightly greater response, and 2, 3 and 5 I.U. produced 95%, 97%, 99% decreases respectively. The same responses were obtained when the ACTH was suspended in 0.5 ml. of gelatine. However, if the volume of gelatine was reduced to 0.1 ml., no significant eosinopenia

occurred. The animals which had received 0.25 and 0.5 ml. showed an average fall in eosinophils of 25% and 51% respectively. It appears that the larger quantities of gelatine (0.5 ml.) produce a stress reaction which lasts throughout the 24 hour period.

Table 2 shows results obtained following the injection into 120 mice

TABLE 2. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH SUSPENDED IN SESAME OIL

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS (0 20 40 60 80 100)
0.05 ml.	0.25	4	6 ± 6.5
	0.5	4	13 ± 13.5
	1.0	10	44 ± 9.2
	2.0	12	92 ± 3.4
0.1 ml.	0.25	9	6 ± 5.6
	0.5	9	28 ± 9.2
	1.0	12	30 ± 11.5
	2.0	8	86 ± 12.5
	4.0	9	89 ± 11.1
0.05 ml.	2.0	12	92 ± 3.4
0.10 ml.	2.0	8	86 ± 12.5
0.20 ml.	2.0	6	68 ± 12.9
0.40 ml.	2.0	6	48 ± 18.1
0.05 ml.	0	6	31 ± 8.2
0.1 ml.	0	6	29 ± 10.5
0.2 ml.	0	6	11 ± 6.6
0.4 ml.	0	3	49 ± 24.9

CONTROLS

of ACTH suspended in sesame oil. It may be seen that a graded dose response is obtained when the ACTH is suspended in 0.05 ml. of oil. Similar results were obtained when a volume of 0.1 ml. was injected, but as the volume was increased to 0.2 and 0.4 ml. responses became less pronounced. The control mice which had received 0.4 ml. of oil had an eosinopenia equal to the group that had received the same volume containing 2 I.U. ACTH. It would appear from the data presented here that the smaller volume of the vehicle produced the most consistently high response.

Table 3 shows the statistical treatment of the data obtained when two strains of mice were injected with 1 and 2 I.U. of ACTH suspended in $\frac{1}{4}$ ml. of gelatine. The first line shows results obtained when two groups of BBF_1 mice were injected with ACTH. The theoretical potency was 100%, the potency ratio obtained was 98.9%, with a standard error of 30%. The index of precision was 0.33 for the 6 sets, indicating a reasonably high degree of accuracy. In line 2 are the results obtained when 11 pairs of mice from each of two strains are compared. In this case the sensitivity ratio was 96% with a standard error of 11%. The index of precision was 0.16, indicating a very high degree of accuracy.

Injections of ACTH and gelatine were made into mice at periods varying

TABLE 3. STATISTICAL RESULTS OBTAINED WHEN STRAIN BBF_1 AND STRAIN 129 MICE ARE INJECTED WITH 1 AND 2 I.U. ACTH IN 0.25 ML. GELATINE

COMPARISON OF BBF_1 AND 129 MICE FOR ASSAY OF ACTH

MICE		RATIO OF	b				
COMPARISON	N	DOSES OF ACTH I.U.	SLOPE	S D	λ	POTENCY RATIO ± S E %	t SLOPE
BBF_1 vs BBF_1	6	$\frac{2}{1}$	− 160	53.3	0.33	98 ± 30	.13
BBF_1 vs 129	11	$\frac{2}{1}$	− 121	20.9	0.16	96 ± 11	.56

from 4 to 30 days after hypophysectomy. A regression formula of $Y = 68 + 67.9 \text{ Log } X$ was obtained for 21 mice injected with $\frac{1}{2}$, 1 and 2 I.U. of ACTH in gelatine. Approximately the same sensitivity was obtained as in normal mice.

Injections of 2 mg. growth hormone in gelatine or sesame oil did not produce a significant eosinopenia over a 24 hour period. On the other hand injections of very toxic oils such as turpentine rsulted in an eosinopenia which persisted for 24 hours or longer. This eosinopenia occurred even in the hypophysectomized animals. For example, injections of 0.05 ml. of turpentine into 7 hypophysectomized animals resulted in the death of 4 animals and an average eosinophil decrease of 76% in the remaining 3.

DISCUSSION

In this paper an attempt has been made to determine the various factors which would be involved in the assay of ACTH in intact mice. Earlier experiments had indicated that a decrease in the number of circulating eosinophils in a relatively short period of 3 or 4 hours was a specific response to the 11-oxycorticosteroids. An assay which is based upon the decrease in eosinophils following an ACTH injection would therefore

measure the amount of 11-oxycorticosteroid hormone released from the adrenal cortex. However, there are a number of factors which make the 4 hour assay impractical in the mouse. Intact animals which have been subjected to handling or mild stress will show an eosinopenia even without the ACTH injections. Furthermore there is a great diurnal variation which must be taken into account. Animals which have been hypophysectomized will also respond to handling and epinephrine injections with a slight eosinopenia (Speirs and Meyer, 1949). In addition it is very difficult to obtain two blood samples from these animals within a period of 4 hours.

However, it was noted in the experiments reported in this paper that following mild stress or injections of non-toxic solvents the eosinophils would completely recover by 16 to 20 hours. The diurnal variation was also found to be consistent. On the other hand, injections of ACTH suspended in gelatine and oil produced an eosinopenia which persisted for 24 hours or longer. These factors suggested that an assay could be devised which would measure the ability of ACTH to depress the number of circulating eosinophils during a 24 hour period.

Injections of 0.5, 1 and 2 I.U. of ACTH were made into intact mice and the following regressions were obtained:

$$Y = 56.02 + 138 \text{ Log } X \text{ (in 0.25 ml. gelatine)}$$
$$Y = 51.1 + 133 \text{ Log } X \text{ (in 0.05 ml. sesame oil).}$$

Determinations of the accuracy of the method were also made (Table 3). These analyses indicated that the assay was reasonably accurate and repeatable with as few as 6 sets of animals. Further comparisons were made between the BBF_1 and the 129 strains. The first strain is known to be highly sensitive to small amounts of 11-oxycorticosteroids and, when adrenalectomized, has been found to be the most satisfactory strain to use for the assay of corticoids. BBF_1 animals are approximately 12 times more sensitive to cortisone acetate than the 129 mice. On the other hand, the 129 strain has been shown to be less responsive to mild stress and handling as measured by a decrease in the circulating eosinophils. It was an entirely unexpected observation that these two strains responded in a similar manner to the long acting ACTH. The 129 mouse is much more docile and easier to handle than the BBF_1 and would therefore make a better assay animal. Other strains are being investigated to determine their sensitivity to ACTH and 11-oxycorticosteroids.

The assay of ACTH by the eosinophil procedure as described above is far less sensitive than the ascorbic acid depletion procedure of Sayers (1948). However, it has the distinct advantage of determining qauntitatively the amount of release of the 11-oxycorticosteroids from the adrenal and the approximate period of time over which the release occurs. For example, very high doses of ACTH in water solutions have little or no eosinopenic

action 24 hours after injection. However, if the ACTH is suspended in gelatine or sesame oil a marked eosinopenia occurs. An assay based upon the duration of eosinopenia would thus be very useful in standardizing and comparing long acting preparations.

Preliminary experiments have indicated that hypophysectomized mice may also be used to assay ACTH solutions. These animals appear to be as sensitive to the ACTH as intact animals. The primary difficulty with this assay in intact animals is that very toxic materials may produce an eosinopenia which persists for 24 hours or longer. Injections of large volumes of slightly toxic materials which are slowly absorbed might also produce a persistent eosinopenia even in hypophysectomized mice. Therefore, care should be taken to run simultaneous controls using identical type and quantity of vehicle.

SUMMARY

The eosinophils in the circulating blood of intact, undisturbed mice were found to have a marked diurnal variation. When counts were performed on groups of 12 mice, an average of 700 eosinophils per cu. mm. was obtained between 8 and 11 A.M. with a rapid decrease to a low value of 155 cells per cu. mm. by 8 P.M. Complete recovery occurred by 8 A.M. the following morning.

Mild stress produced a marked eosinopenia in 3 to 6 hours followed by an eosinophilia. There was a recovery in the level of circulating eosinophils within 24 hours.

Injections of ACTH in saline solution produced an eosinopenia which was almost identical to that produced by mild stress. However, when the ACTH was suspended in gelatine or sesame oil, the period of eosinopenia was prolonged up to 24 hours or more.

These observations suggested that an assay for ACTH could be performed in either intact or hypophysectomized mice. The per cent decrease in eosinophils over a 24 hour period was found to be proportional to the quantity of ACTH injected. Statistical analysis of the data indicated that the regression was $Y = 56.02 + 138\ X$ when 0.5, 1 and 2 I.U. of ACTH was suspended in 0.25 ml. gelatine and injected into intact mice. The index of precision of 6 sets of animals injected with 1 and 2 I.U. ACTH in gelatine was found to be 0.33.

Two strains of mice tested for this assay, the BBF_1 hybrids and the 129 mice, were found to give almost identical responses. The index of precision of 11 sets of animals was found to be 0.16.

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THE ACTION OF DESOXYCORTICOSTERONE ACETATE ON THE MAMMARY GLAND OF THE IMMATURE OVARIECTOMIZED RAT

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DESOXYCORTICOSTERONE acetate (DCA) exerts a progesterone-like action on the uterus (Van Heuverswyn, *et al.*, 1939a; Engle, 1941; Masson, 1943) and mammary gland (Mixner and Turner, 1942; Trentin and Turner, 1948; Speert, 1940) of several laboratory animals. It also shows estrogen-like activity on the mammary gland (Van Heuverswyn, *et al.*, 1939b; Leonard and Reece, 1942.) However, no comprehensive study of its action on the mammary gland of the rat has been undertaken. The experiments reported here were designed to elucidate the action of DCA on the mammary gland of the immature ovariectomized rat, alone, and in combination with estradiol or progesterone.

MATERIALS AND METHODS

Twenty-four day-old female albino rats were ovariectomized. Treatment was begun on the 29th day with daily subcutaneous injections of the hormones in sesame oil with the exception of the highest dose of DCA which was administered in propylene glycol.[3] A standard daily dose of 0.1 γg. estradiol and of 4.0 mg. progesterone was injected. The doses of DCA were varied from 1.0 to 10.0 mg. daily. The rats were treated for 12 days. Autopsy was performed the day after the last injection. The right abdominal-inguinal mammary glands of each animal were removed, fixed in Bouin's solution, stained with Mayer's haematoxylin, and stored in methyl salicylate for later study as whole mounts.

The areas of the glands were traced onto weighing paper and later measured with a Keuffel and Esser planimeter. The glands were trimmed of peripheral fat and connective tissue and weighed on a triple-beam balance. Lobulo-alveolar growth was rated on a similar scale. The uteri were also trimmed, split, blotted on bibulous paper, and weighed for comparative study.

RESULTS

Daily doses of 2 and 5 mg. DCA resulted in glands with ducts which were dilated and extended, and buds which were expanded (Table 1). There was no alveolar formation. Mammary gland areas were somewhat smaller

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[3] The steroids used in these studies were generously supplied by the Schering Corporation, Bloomfield, New Jersey.

than in the controls. The glands resembled histologically those of animals treated with estradiol alone. At a daily dose of 10 mg., DCA had no effect on mammary duct growth. The glands resembled those of untreated castrated controls, in spite of the fact that uterine growth was almost as great as that of rats treated with estradiol.

TABLE 1. INFLUENCE OF DCA OR ESTROGEN ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.²)	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
None	5	64	105	6.0±0.37	0.29±0.070	0/5	32.2
0.1 γg. Estrogen	4	63	102	6.1±0.35	0.36±0.050	0/4	128.5
2 mg. DCA	3	65	110	5.0±0.47	0.24±0.015	0/3	Atrophic
5 mg. DCA	3	69	113	3.9±0.32	0.26±0.040	0/3	Atrophic
10 mg. DCA	2	50	93	5.9	0.36	0/2	93.5

Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

It is well known that estradiol and progesterone synergize to induce lobulo-alveolar growth. Since DCA apparently exhibited estrogenic properties in the preceding experiments, further experiments were designed to determine whether DCA would substitute for estradiol in this synergistic combination (Table 2). Animals injected with 4.0 mg. of progesterone daily showed glands with some duct and end bud expansion, and dubious lobulation. In all rats receiving 0.1 γg. estradiol and 4.0 mg. progesterone daily, the ducts and end buds were expanded, and excellent lobulation occurred.

TABLE 2. INFLUENCE OF PROGESTERONE, DCA AND PROGESTERONE, AND ESTROGEN AND PROGESTERONE ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.²)	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
4 mg. Progesterone	3	71	114	7.8±0.39	0.36±0.048	1/3	53.3
4 mg. Progesterone + 2 mg. DCA	3	54	92	6.8±0.58	0.28±0.011	0/3	45.0
4 mg. Progesterone + 5 mg. DCA	3	62	106	6.3±0.43	0.31±0.020	0/3	84.2
4 mg. Progesterone + 0.1 γg. Estrogen	3	64	101	7.2±0.67	0.35±0.057	3/3	114.4

(Fig. 1). A combination of 4.0 mg. progesterone and 2 mg. DCA daily led to expanded ducts and buds, and, in two out of three rats, doubtful alveolar development. With a combination of 4.0 mg. progesterone and 5 mg. DCA daily, there was spotty duct extension and dilation, slight bud expansion, and no lobulation.

Rats given 5 mg. of DCA daily, in addition to progesterone, had uterine weights which were around 50% above rats receiving progesterone alone. Two mg. DCA failed to effect this increase. In rats treated with estradiol and progesterone, average uterine weight was more than 100% increased over the progesterone controls.

The next step was to determine whether or not DCA would exhibit any progesterone-like activity in combination with estradiol. The possibility of any additive effects with the estrogen was also looked for. Reference to Table 3 shows that 0.1 γg. estradiol and 2.0 mg. DCA daily did not elicit any lobulo-alveolar growth, while 0.1γg. estradiol and 5.0 mg. DCA led to some lobulation in four of five rats: three gave a "plus one" reaction, and one gave a "plus two" reaction. Mammary areas showed little change

TABLE 3. INFLUENCE OF ESTROGEN-DCA COMBINATIONS ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area ($cm.^2$)	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen + 2 mg. DCA	4	55	94	5.1 ± 0.44	0.34 ± 0.041	0/4	120.3
0.1 γg. Estrogen + 5 mg. DCA	5	62	114	6.2 ± 0.18	0.33 ± 0.059	4/5	111.1
0.1 γg. Estrogen + 10 mg. DCA	5	42	92	7.5 ± 0.63	0.31 ± 0.029	2–3/5	111.3
Propylene glycol	2	—	—		Castrate Atrophy		—

from those of rats receiving estrogen alone (Table 1). Estradiol, 0.1 γg., and DCA, 10 mg., daily led to a "plus three" in one and a spotty "plus two" reaction in two of five rats. One of the better responses is seen in Figure 2, comparable to that obtained with 0.1 γg. estradiol and 4.0 mg. progesterone (Fig. 1). Mammary areas were increased in this group, comparable to those of rats treated with estradiol and 4.0 mg. progesterone (compare with Table 2). Propylene glycol alone had no effect on the mammary glands, which were identical in appearance with those of the ovariectomized, uninjected rats. It is evident from these results that DCA at a dose of 5 mg. daily exhibits only a slight progesterone-like activity in the syner-

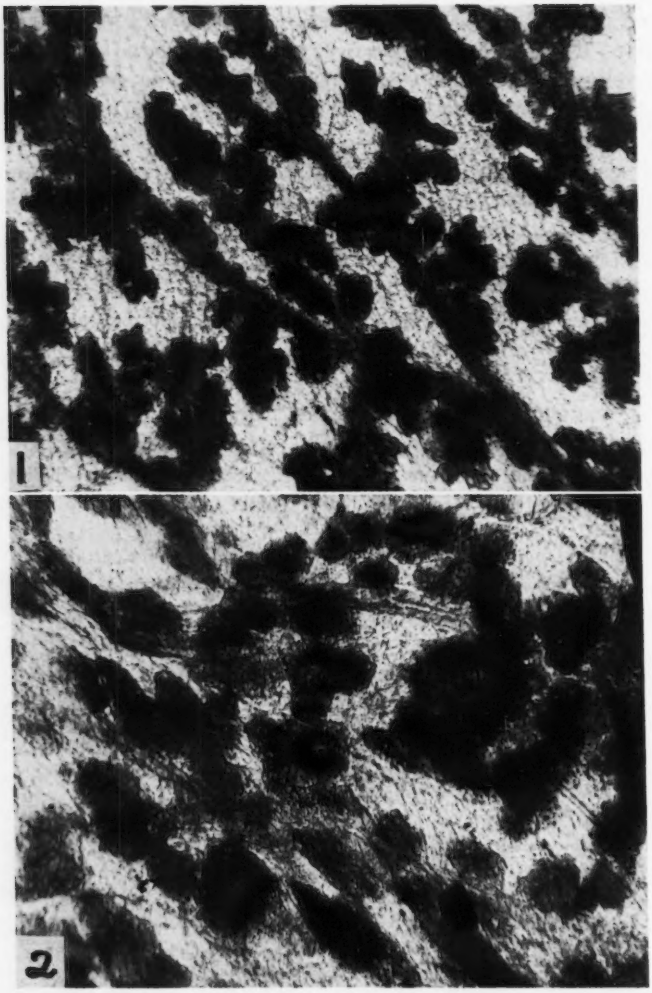

FIG. 1. Mammary gland of rat injected with 0.1 γg. estradiol and 4.0 mg. progesterone for 12 days.

FIG. 2. Mammary gland of rat injected with 0.1 γg. estradiol and 10.0 mg. DCA for 12 days.

gistic reaction with estradiol causing lobulation. Doubling the daily dose of DCA produced little additional effect on mammary lobulation. Nor could any additive effects with the estrogen be detected, either on the mammary duct system or on uterine weight.

The possibility remained that DCA, because of structural similarity to

progesterone, might exhibit an additive or antagonistic action to the lobulo-alveolar induction by 4.0 mg. progesterone and 0.1 γg. estradiol daily. DCA, in doses of 1.0, 2.0, and 4.0 mg. daily failed to exert any additive or antagonistic effects, either on lobulo-alveolar growth, duct structure, or total area (compare results in Table 4 with those in Table 2). Uterine weights were unaltered by the superimposed DCA treatment.

TABLE 4. INFLUENCE OF DCA ON MAMMARY GROWTH ELICITED BY ESTROGEN AND PROGESTERONE

Daily treatment	Number of rats	Body weight (gm.)		Area ($cm.^2$)	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen+ 4 mg. Progesterone +2 mg. DCA	2	77	116	6.9	0.33	2/2	111.7
0.1 γg. Estrogen + 4 mg. Progesterone +2 mg. DCA	4	64	106	7.2±0.81	0.37±0.054	4/4	102.2
0.1 γg. Estrogen + 4 mg. Progesterone +4 mg. DCA	4	58	101	6.8±0.60	0.33±0.038	4/4	114

DISCUSSION

DCA has mammary duct-stimulating activity in the mouse in doses of 0.5 mg. every other day (Van Heuverswyn, *et al.*, 1939b) and in the partially-hypophysectomized rat (Leonard and Reece, 1942). The present experiments furnish evidence that it exerts both an estrogen-like and a progesterone-like action on the mammary gland of the immature ovariectomized rat. Even though DCA is estrogenic when administered alone, it fails to substitute for estradiol in the synergistic reaction with progesterone to bring about lobulo-alveolar development. In this respect, its estrogenic activity is peculiar, and perhaps, atypical.

The amount of DCA required to produce even slight lobulo-alveolar growth in the rat is much greater than that reported by Mixner and Turner (1942) to produce lobulo-alveolar growth in the mouse. In their experiments, DCA was approximately one-third as potent as progesterone. An exact ratio of DCA to progesterone cannot be determined from the present data. However, it must be pointed out that the amount of progesterone, injected with estrogens, required to produce optimal lobulation is also greater in the rat (Curtiss, 1949, Smith, 1952, in preparation).

The progestin-like activity of DCA in these experiments may be due in part to a conversion of the injected DCA to progesterone, similar to that which has been reported for the human and chimpanzee (Horwitt, *et al.*, 1944), macaque (Zarrow, *et al.*, 1950), rabbit (Hoffman, *et al.*, 1943) and mouse (Hooker and Forbes, 1949). However, this conversion, or other metabolic pathways of DCA have not been demonstrated in the rat. Consequently, we have no hint as to what extent DCA itself or the metabolites of DCA are responsible for the mammotropic effects seen in the present experiments.

Other lines of evidence have implicated the adrenal cortex in mammary growth processes. These are the several reports that adrenalectomy affects mammary growth (Butcher, 1939; Reeder and Leonard, 1944; Trentin and Turner, 1947; Johnston and Smithcors, 1948; Cowie, 1949; Smith, 1951). In the last-mentioned study, lobulo-alveolar growth was unaffected by adrenalectomy, and the reduced duct response to the ovarian steroids and relaxin was restored by administration of DCA, or other corticoids. Other reported effects have varied widely, from slight stimulation to partial suppression of growth, but are not completely unreconcilable.

If the DCA-type compounds play more than a sustaining role for mammary growth in the rat, endogenous production must be enormous, and might even approach a toxic level. Three of nine rats receiving the highest dose of DCA, 10 mg. daily, died during the course of treatment, while rats receiving the propylene glycol alone remained healthy. These results suggest that this is not only a maximal hormonal dose, but also approaches a toxic pharmacological dose for this species.

It might be expected that since DCA resembles progesterone structurally, but does not itself exert the consistent lobulating activity of progesterone, it would interfere with the optimal growth elicited by estradiol and progesterone. The possibility that the doses of DCA were too low, or the dose of progesterone was relatively high needs further scrutiny. However, with the gravimetric ratios used: 1:4, 1:2, and 1:1, no evidence for any inhibitory effect was obtained. Nor was the reverse of this, an additive or synergistic effect, observed.

SUMMARY

Desoxycorticosterone acetate (DCA) alone exerts a duct-stimulating effect on the mammary gland of the immature ovariectomized rat. Alone, or in combination with progesterone, it does not effect any lobulo-alveolar growth. Five mg. of DCA daily, given simultaneously with estradiol, exhibits a slight lobulating action, but doubling the dose of DCA to 10 mg. produces little additional lobulation. No antagonistic or additive effect

of DCA upon the synergistic reaction of estradiol and progesterone in stimulating lobulation could be detected.

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EVIDENCE FOR A DUAL ACTION OF PARATHYROID EXTRACT IN MAINTAINING SERUM CALCIUM AND PHOSPHATE LEVELS[1]

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THE primary mechanism through which the parathyroid glands influence mammalian calcium and phosphate metabolism is still an unsettled problem. The results of past experiments have led to two divergent views regarding the action of the parathyroid hormone: one view maintaining that the principal role of the hormone is the regulation of the osteoblastic and osteoclastic activity of bone tissue, while the other view claims its action to be primarily in controlling the phosphate threshold of the kidney. For a recent review of the work the reader is referred to Greep (1948).

While much of the recent work has been in favor of the kidney threshold theory, evidence has also accumulated suggesting an extra-renal function of the hormone. Considerable work has been reported showing the influence of the parathyroids on bone metabolism both before and after removal of the kidneys (Selye, 1942; Ingalls *et al.*, 1943; and Barnicot, 1948). In addition, Monahan *et al.* (1944) produced a fall in serum calcium in dogs parathyroidectomized after total nephrectomy. Stoerk (1943) prevented a fall in serum calcium levels of rats after total nephrectomy and parathyroidectomy by administration of parathyroid extracts. Similarly, Stewart and Bowen (1951) produced a rise in blood calcium values after nephrectomy in both normal and parathyroidectomized dogs. On the other hand, Tweedy *et al.* (1947, 1950) failed to find any influence of this gland on radio-phosphorus and radio-calcium distribution in nephrectomized rats.

The purpose of this study is to present evidence that parathyroid extract may produce its primary influence on serum phosphate and calcium levels by independent and separate mechanisms.

MATERIALS AHD METHODS

More than 150 male Wistar rats, weighing between 200 and 250 grams were used in these experiments. The parathyroids were removed individually after the method of

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Richter and Birmingham (1941). Nephrectomies were performed in two stages, the left kidney being removed three to four days prior to the right. Since the rat is very resistant to the removal of the parathyroids, possibly due to the presence of accessory tissue, the test for hypoparathyroidism was a drop in serum calcium to below 7 mg.% and the appearance of a partial tetanic response in the animal. Animals not showing the external nervous manifestations were discarded.

Parathyroid extract[3] was administered subcutaneously in a water solution, each injection containing 50 USP units. Four injections were given over a 24 hour period and the animals were killed four to six hours after the last injection. Blood was removed by heart puncture prior to killing. Serum calcium levels were determined by the method of Clark and Collip (1934); serum phosphates as described by LePage (1949).

Except where otherwise indicated, the experimental procedure was as follows: On Day 1, the parathyroids were removed. The left kidney was removed either on the same or the following day. The animals were maintained on stock diet during the period of recuperation. The actual experiment began with the removal of food in the early evening of Day 4. Eighteen hours later, on Day 5, the parathyroidectomized animals which did not show external manifestations of parathyroid deficiency were discarded, and the remaining animals were divided into the various experimental groups. At this time the second kidney was removed and/or the first injection of parathyroid extract was given. Additional injections were given six and twelve hours later and on the morning of Day 6. The experimental procedures were terminated by bleeding the animals on the afternoon of Day 6. Control groups were included in each experiment and were treated identically in respect to food, removal of kidneys, etc.

RESULTS

The animals used in these experiments were divided into ten groups. Those in the first five groups had intact parathyroid glands, while the remaining animals were parathyroidectomized. A brief description of the experimental treatment of each group is given in Table 1, and the results are summarized in Table 2.

TABLE 1

Group	Parathyroid glands intact	Parathyroid glands removed	Kidneys removed		Parathyroid administration
			Left	Both	
1	*				
2	*		*		
3	*		*		*
4	*			*	
5	*			*	*
6†		*	*		
7		*	*		
8		*	*		*
9		*		*	
10		*		*	*

* For time sequence of operations and parathyroid extract administration see Materials and Methods.

† Killed at the time nephrectomy was completed in other animals; these serve as a baseline for further changes in serum and calcium values following complete nephrectomy.

[3] Lilly's Solution of Parathyroid Extract.

TABLE 2. SERUM PHOSPHATE AND CALCIUM VALUES

Group	Calcium		Phosphate	
	No. of animals	Value ±S.E.	No. of animals	Value ±S.E.
A. *Intact parathyroids*				
1. Stock Animals	9	11.7±0.18	6	7.7±0.12
2. Controls	21	11.2±0.19	6	5.4±0.34
3. PTH*	7	12.1±0.40	7	5.0±0.31
4. Nephx†	11	11.6±0.32	7	14.6±0.61
5. Nephx-PTH	5	11.3±0.42	5	14.2±0.56
B. *Parathyroidectomized*				
6. PTX‡ only	21	5.7±0.19	4	15.3±1.12
7. Prenephx	8	6.7±0.31	7	10.6±0.43
8. PTH	8	10.1±0.57	8	6.3±0.41
9. Nephx	18	5.3±0.23	11	17.7±0.43
10. Nephx-PTH	22	7.8±0.25	16	17.4±0.44

* Injected with parathyroid extract.
† Nephrectomized.
‡ Parathyroidectomized.

COMPARISON OF SERUM CALCIUM VALUES

The average serum calcium value for the normal rat has been found by us to be over 11 mg.% (Group 1). In the animal with intact parathyroids, this value is maintained at least up to 42 hours of starvation (Group 2), and in the totally nephrectomized starved rats is still constant 36 hours after removal of the last kidney (Group 4).

The administration of parathyroid extract for 24 hours to the normal but starved rat produced a questionable rise in the serum calcium value (Group 3). Previous experiments had shown that if this dosage was continued for an additional two days it would, in normal fed rats, raise the serum calcium level to approximately 15 mg.%. As noted by Tweedy *et al.* (1950), no increase was seen in the serum calcium level of nephrectomized rats treated with the hormone for twenty-four hours (Group 5).

In the parathyroidectomized rat it was found necessary to starve the animal in order to produce a marked drop in the serum calcium. Up to six days after removal of the parathyroids, animals maintained on a normal diet usually had a serum calcium value in the low normal range and only rarely did this drop as low as 9 mg.%. However, if food is removed, as done in these experiments on Day 4, the calcium values drop rapidly and 18 hours later will be found to be approximately 6.7 mg.% (Group 7). 36 to 42 hours after removal of the food, the value will have dropped to 5.5 mg.% (Group 6). As seen in Group 9, nephrectomy does not appear to alter significantly this chain of events.

If, after starvation for 18 hours, parathyroid extract was administered to the parathyroid deficient rat, the serum calcium values rose rapidly and

24 hours later were found to be in the low normal range (Group 8). When total nephrectomy was completed prior to injection of the hormone, a rise in serum calcium was still produced though not as marked (Group 10). Thus, a further decrease was not only prevented, but an actual increase in the serum values was observed as shown by comparison of groups 7, 9, and 10.

COMPARISON OF SERUM PHOSPHATE VALUES

The serum phosphate level of normal animals has been found by us to be approximately 7.5 mg.% (Group 1). This value is, however, markedly affected by environmental conditions, particularly diet (Tepperman *et al.*, 1947). Starvation for 36 to 42 hours caused this value to drop to 5.4 mg.% (Group 2). Parathyroidectomy caused a rapid rise in the serum values. After the 18 hour starvation period, the phosphate level of those animals showing external manifestations of parathyroid deficiency had risen to approximately 10.6 (Group 6), and by 36–42 hours was up to 15.3 mg.% (Group 5).

Nephrectomy, in the normal animal, produced a rise in serum phosphate to approximately the same value as the non-nephrectomized parathyroid deficient animal (Group 4). Nephrectomy, however, increased only slightly the already high phosphate level of the parathyroidectomized rat (Group 9).

At the dosage used in these experiments, parathyroid extract treatment had no significant effect on the serum phosphate in the normal animal (Group 3). In the parathyroid deficient animal, the hormone caused a rapid drop in the serum values to a value approaching the normal condition (Group 8). Nephrectomy apparently negated this effect of the hormone entirely (Groups 9 and 10).

DISCUSSION

Since Collip *et al.* (1925) first discovered the influence of the parathyroids on calcium and phosphorus metabolism, it has become well-established that as a result of parathyroid removal the serum calcium level rapidly falls, accompanied by a corresponding increase in the serum phosphate value. Very little emphasis, however, has been placed on the observations, which have been shown before and confirmed by these studies, that the sequence of changes in the serum calcium levels is not influenced by total nephrectomy performed either at or subsequent to the removal of the parathyroids; and that only minor further increases in the phosphate serum values are produced by such removal of the kidneys.

It has also been well established that, in the hypoparathyroid animal in which low serum calcium and high phosphate levels are present, injection of

parathyroid extract produces an immediate increase in the excretion of phosphate by the kidneys, accompanied by a lowering of the phosphate and raising of the calcium serum values. Removal of the kidneys prior or during this sequence of events has a marked influence only on the phosphate changes. All effect of the hormone on the phosphate levels seemingly is lost; however, the calcium values *will* continue to rise though the rate is somewhat restricted.

Careful consideration of these data and those presented by the investigators referred to in this work points very strongly to the facts that calcium and phosphate serum values can vary independently of each other; that normal calcium values can be maintained in the presence of phosphate values two and a half to three times their normal value, and that the effect of parathyroid extract on serum calcium of the parathyroid deficient rat is not negated by the removal of the kidneys.

In the light of these facts, one cannot see how any theory of the sequence of events produced by parathyroid extract can be tenable which makes renal excretion of phosphorus the only means by which the hormone can influence calcium metabolism, though indirect influences by means of changes in the phosphate levels very probably occur (Albright and Reifenstein, 1948).

At the present time there appears to be two alternatives: one which would conclude that the hormone exerts its influence directly on only one of these ions, and that the indirect influence on the other is caused, not only by the solubility product of these two ions in the blood, but also by some other extra-renal influence either in the bones or elsewhere; or that the hormone has a dual action, controlling phosphate primarily through its renal effect, and calcium primarily through an extra-renal influence.

Due to the wealth of data showing the influence of the parathyroids on renal excretion of phosphate and to the widely fluctuating serum phosphate levels possible in the presence of a constant calcium value, the latter alternative is considered to be more likely.

SUMMARY

The influence of nephrectomy upon the action of parathyroid extract has been studied in rats. This extract was administered to normal and parathyroid deficient rats which had been nephrectomized in a two-stage operation. Both serum phosphate and serum calcium values were determined. Nephrectomy appeared to nullify all effects of parathryoid extract upon serum phosphate levels. However, the effect of the extract on serum calcium, though somewhat suppressed, was still apparent. Not only did the parathyroid extract prevent a further decrease in serum calcium in the nephrectomized parathyroid-deficient rat, but when 200 USP units were

administered over a 24 hour period following removal of the last kidney, a definite rise in serum calcium was noted. Due to these observations, it is concluded that for its normal function, the parathyroid extract has a direct and independent effect on both calcium and phosphate metabolism.

ADDENDUM

Attention is called to the recent publication by Stewart, G. S. and H. F. Bowen (*Endocrinology 51:* 80. 1952) which appeared since submitting this manuscript. These authors are suggesting that the phosphate excretion factor of the parathyroid gland extract is an artifact, leaving the calcium mobilizing factor the primary function of the parathyroid glands.

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THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE RENAL TUBULAR REABSORPTION OF PHOSPHATE AND THE EXCRETION OF TITRATABLE ACID AND POTASSIUM IN DOGS[1]

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IT HAS been demonstrated that the renal tubules of dog and man are capable of reabsorbing a fixed and limited quantity of phosphate per unit of time when presented with an excess in the glomerular filtrate (Pitts *et al.*, 1944; Schiess *et al.*, 1948). Since absorptive capacity is independent of glomerular filtration rate over a fairly wide range (Ayer *et al.*, 1947) the renal mechanism which handles phosphate, like that which handles glucose, exhibits a true Tm relationship. More recently, it has been observed, in man, that an increased renal excretion of phosphate follows the administration of cortisone and ACTH. Moreover, negative phosphate balance, depletion of intracellular stores of phosphate and demineralization of bone have been observed in conditions of hyper-adrenocorticism (Albright 1942, Eliel *et al.*, 1951; Ingbar *et al.*, 1951; Pearson *et al.*, 1950; Freeman *et al.*, 1950; Soffer *et al.*, 1950). Although logically inferred, it has not been clearly defined that this increment in phosphate excretion results from an alteration in the renal tubular capacity for phosphate reabsorption consequent upon an excess of adrenal hormone. Accordingly the series of experiments described below was performed to determine the effects of cortisone and desoxycorticosterone on the renal tubular reabsorption of phosphate. Since exchange of hydrogen and potassium ions for sodium bound by urinary buffers plays a significant role in sodium conservation (Berliner, 1951, Pitts 1950), the effects of these steroids on titratable acid and potassium excretion were observed simultaneously. These experiments show that cortisone depresses the renal tubular reabsorptive capacity for phosphate, whereas desoxycorticosterone is without effect. On the other hand, no clear cut effects of either steroid could be demonstrated on the renal mechanisms involved in the excretion of titratable acid and potassium.

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METHODS

In all, 21 experiments were performed on 4 normal and 2 adrenalectomized dogs, both untreated and treated with cortisone or desoxycorticosterone.[3] Prior to the experiments on adrenalectomized dogs, hormone treatment was discontinued for 5–7 days and the animals were maintained with 0.6% salt solution *ad lib.* during this interval. In order to define the maximal tubular capacity for reabsorption of phosphate, the plasma phosphate level and thus the amount presented to the renal tubules were increased by the infusion of neutral sodium phosphate. In some of the experiments the plasma phosphate level was elevated in stepwise fashion; in others a constant infusion of phosphate was maintained. Cortisone, in amounts of 50 to 75 mgm., or desoxycorticosterone, 2 to 5 mgm., was administered intravenously either prior to, or during the course of the experiment. Cortisone acetate was given as a microcrystalline suspension in saline. Dexosycorticosterone was given as the crystalline preparation dissolved in sesame oil and homogenized with saline. Creatinine, which was used to measure the rate of glomerular filtration, was infused with the phosphate in amounts sufficient to maintain optimum plasma levels. Sodium and potassium concentrations of plasma and urine were determined with an internal standard flame photometer. Creatinine was determined colorimetrically by the method of Bonsnes (Bonsnes, 1945). Titratable acid excretion was determined by electrometric titration as described in a previous communication (Pitts *et al.*, 1945). Phosphate was determined on trichloracetic acid filtrates of plasma and diluted urine by the method of Fiske and Subbarow (1925) as modified by Pitts (1933).

RESULTS

Table 1 summarizes the data of three typical experiments performed on three dogs and demonstrates the effect of the administration of cortisone or desoxycorticosterone on the reabsorption of phosphate. In these experiments constant infusions of neutral phosphate were instituted 30 min. prior to the first control period and maintained throughout the experiment. Following two control periods of twenty minutes, the steroid was given and the effect upon phosphate reabsorption, potassium and titratable acidity measured over a period of 60–80 minutes. The experiment at the top of the table illustrates the decrease in tubular reabsorption of phosphate which follows the injection of 50 mg. of cortisone in a normal dog. This occurs within a thirty-minute period following steroid administration. The second experiment shows that desoxycorticosterone has no effect on the renal tubular reabsorption of phosphate. The third experiment demonstrates that cortisone caused a decrease in phosphate reabsorption in an adrenalectomized dog maintained without hormone substitution therapy for six days which was similar to that seen in the normal animal.

Figure 1 shows graphically the average change in phosphate Tm resulting from adrenal steroid injection in four normal dogs. Each box represents the average phosphate reabsorption in mM./min. over a period of 90 to

[3] We are greatly indebted to the Schering Corporation for the generous supply of desoxycorticosterone used in these experiments.

100 minutes in untreated dogs and following the administration of cortisone or desoxycorticosterone. The average phosphate Tm was calculated by averaging the phosphate reabsorbed in 3–5 different experiments on each dog. From this, it can be seen that cortisone significantly reduced phosphate reabsorption whereas desoxycorticosterone had no such effect.

TABLE 1. THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE PHOSPHATE REABSORPTION IN TWO NORMAL DOGS (1 AND 2) AND ONE ADRENALECTOMIZED DOG (3)

Urine flow cc./min.	Glomerular filtration rate,	Plasma		Phosphate			Excreted	
		Phosphate mM/L	Potassium mM/L	Filtered	Excreted	Reabsorbed	Titratable acid	Potassium
				mM/min.			mM/min.	
				1				
				Control				
1.9	75.7	4.26	3.64	.322	.211	.111	.046	.086
2.31	75.5	5.29	3.14	.405	.305	.100	.056	.094
				Cortisone				
3.7	74.8	5.9	3.14	.441	.379	.062	.056	.073
4.8	75.5	6.23	3.0	.470	.430	.040	.075	.089
6.2	81.2	6.48	2.7	.526	.476	.050	.080	.131
				2				
				Control				
1.2	103	4.07	3.85	.419	.262	.157	.052	.092
1.4	94	4.29	3.85	.403	.266	.137	.050	.072
				Desoxycorticosterone				
3.3	94	4.65	3.85	.435	.304	.131	.107	.063
4.1	102	4.77	3.8	.487	.343	.144	.095	.108
3.8	106	4.80	2.6	.509	.359	.150	.085	.139
				3				
				Adrenalectomized				
				Control				
2.6	68	5.96	5.5	.405	.203	.202	.159	.063
3.4	69	6.07	5.8	.418	.224	.194	.152	.082
				Cortisone				
2.9	61	5.84	5.5	.356	.228	.128	.110	.128
3.0	61	5.42	5.0	.330	.217	.113	.097	.135
2.7	66	4.95	4.5	.327	.204	.123	.095	.142

TITRATABLE ACID AND POTASSIUM EXCRETION

Figure 2 shows the increase in potassium and titratable acid excretion which occurs as increasing amounts of phosphate are filtered and thus excreted, under normal circumstances and under the influence of cortisone. In the upper part of the figure the sum of potassium and titratable acid, as a measure of sodium conservation, is plotted as a solid line. In the lower part of the figure is shown the increment in sodium excretion which occurs simultaneously with the increased excretion of titratable acid and potas-

sium. In this pair of experiments the amounts of these substances excreted are seen to be somewhat increased following cortisone, especially when smaller amounts of phosphate were filtered. We have presumed this to be related to the decrease in phosphate Tm. The resulting increase in phosphate excretion per unit filtered led to an increase in sodium, potassium

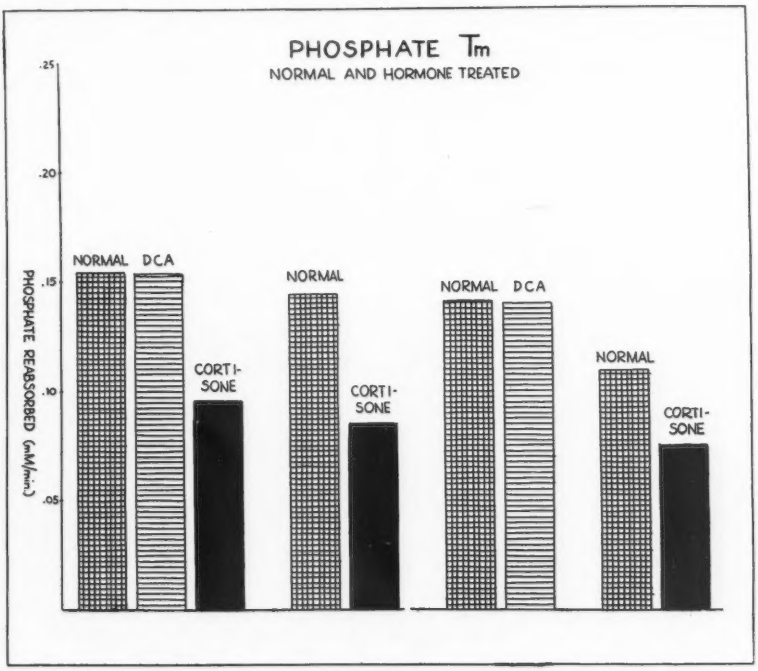

FIG. 1. Effects of cortisone and desoxycorticosterone on the average phosphate reabsorption in four normal dogs.

and hydrogen ion excretion. However this has not been a consistent finding. In all animals, the general pattern of titratable acid and potassium excretion was not significantly altered by the administration of adrenal steroid. Further, the response of the adrenalectomized salt-maintained animal did not differ from the normal when the animal was presented with a phosphate load. Reference to Table 1 shows that the adrenalectomized animal excretes considerable amounts of titratable acid and potassium following administration of phosphate. Similar results were obtained in 6 experiments on 2 adrenalectomized dogs.

Figure 3 illustrates further that no specific quantitative differences in titratable acid excretion could be attributed to the influence of adrenal

steroid. In this figure is shown the rise in titratable acid excretion which occurs as increased amounts of phosphate are excreted in the normal and adrenalectomized dog, both with and without adrenal hormone. No consistent differences were noted between normal animals and those subjected to adrenalectomy, nor was the excretion of hydrogen ions effected by cortisone or desoxycorticosterone.

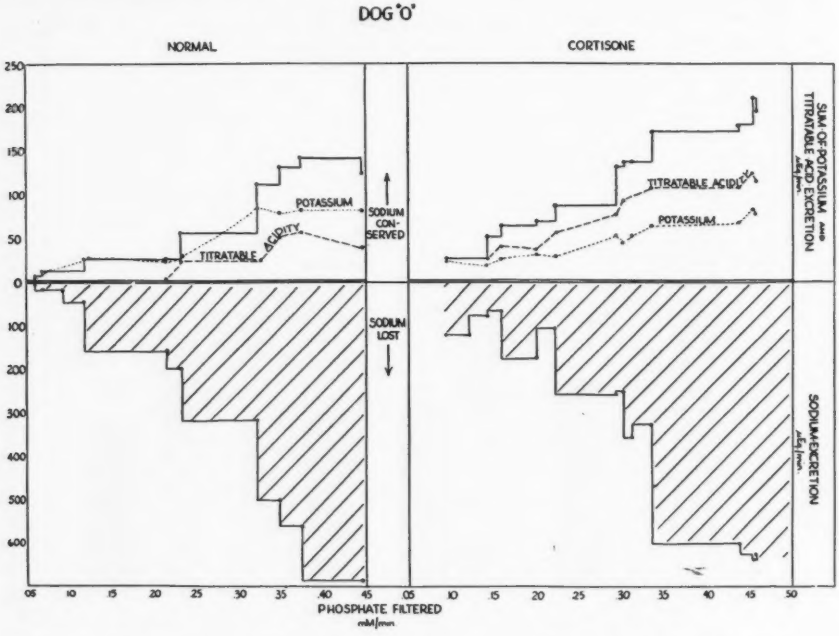

FIG. 2. Sodium, potassium and titratable acid excretion during phosphate loading in a dog with and without cortisone.

DISCUSSION

From the data presented above, it is evident that the depression of phosphate Tm following cortisone administration may be a factor in bringing about the negative phosphate balance observed following the administration of cortisone or ACTH. The increase in the renal excretion of phosphate if continued sufficiently long could account for intracellular depletion of phosphate.

Although other factors may be operative in bringing about the alterations in phosphate metabolism observed in hyperadrenocorticism, these experiments do not elucidate the nature of these mechanisms; nor was any attempt made in these experiments to compare the relative significance of

a reduction in phosphate Tm to other factors known to influence phosphate metabolism.

Since the adrenalectomized rat and the Addisonian patient have been reported to show defects in ammonium and titratable acid excretion, it might be expected that the adrenalectomized dog would display a renal deficit in the exchange of hydrogen and potassium ions for sodium (Loeb *et al.*, 1933; Pitts, 1950; Stahl *et al.*, 1936). Such was not found to be the

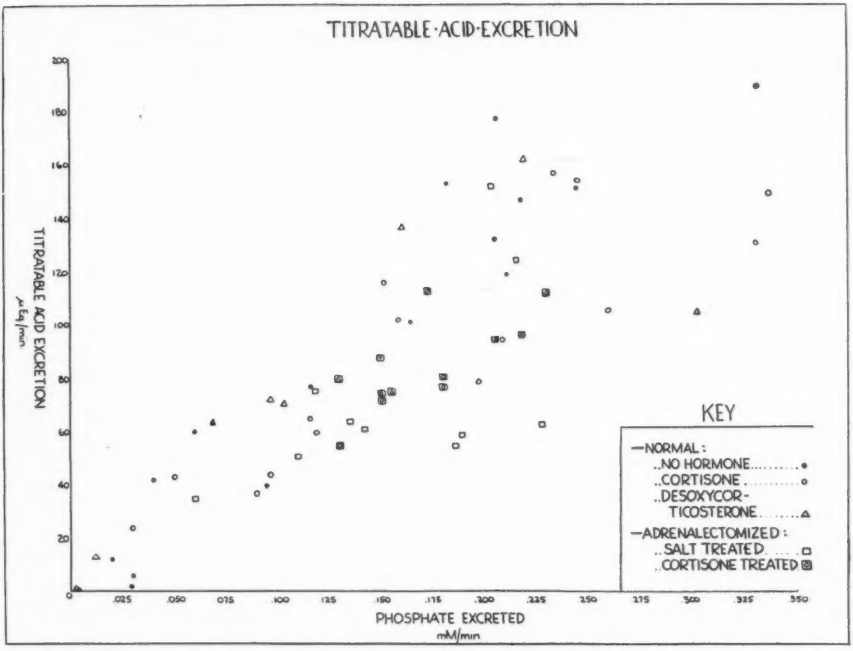

FIG. 3. Titratable acid excretion plotted as a function of phosphate excretion in normal, adrenalectomized and hormone-treated dogs.

case in our experiments. When presented with a phosphate load, the excretion of titratable acid and potassium did not differ significantly from that seen in normal animals. Further, this response was not modified by cortisone. In fact, no clear cut effects of either cortisone or desoxycorticosterone could be demonstrated upon the excretion of these substances per se: any alterations which did occur appeared to be secondary to those alterations in phosphate excretion which followed cortisone administration. From this, we infer that cortisone does not exert a major influence directly on the renal tubular mechanisms involved with the exchange of hydrogen and potassium for sodium in the dog.

CONCLUSIONS

(1) The renal tubular reabsorptive capacity for phosphate is decreased following the administration of cortisone in intact and adrenalectomized dogs. (2) Desoxycorticosterone does not depress the phosphate Tm. (3) The increase in excretion of titratable acid and potassium, which occurs as increasing amounts of phosphate are excreted, is not significantly altered by administration of cortisone or desoxycorticosterone. (4) Adrenalectomized animals maintained on sodium chloride show the same increase in the excretion of titratable acid and potassium following phosphate loading, as that seen in intact animals given a similar phosphate load.

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CYTO-PHYSIOLOGIC ASPECTS OF THYROTROPHIC HORMONE SECRETION IN THE GOITROUS GUINEA PIG*

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THE assignment of cell types to specific hormone secretions in the adenohypophysis continues to thwart proper understanding of the mechanisms underlying thyroid-hypophyseal interplay. Earlier opinion (Severinghaus, 1937) favored the pituitary acidophile as the source of thyroid stimulating hormone (TSH) but recent investigations, based largely on the rat, more clearly implicate the basophiles (Griesbach and Purves, 1945; Brolin, 1945; Goldberg and Chaikoff, 1949; and Halmi, 1950). Purves and Griesbach (1951) moreover, have applied histochemical procedures to the rat adenohypophysis which allegedly discriminate between thyrotrophic and gonadotrophic hormone producing cells routinely classed as basophiles. Despite the apparent relationship between thyroid activation and pituitary basophilia (D'Angelo, 1941; Griesbach, 1951), cellular localization of TSH production in the hypophysis is not satisfactorily established. The general failure to demonstrate increased content of TSH in the rat pituitary after procedures (thyroxine deficiency, cold) which presumably activate the thyrotrophic mechanism clouds interpretation of its morphology (Kuschinsky, 1935; Turner and Cupps, 1940; Griesbach and Purves, 1943). The need for histophysiologic studies of thyroid-pituitary interaction in species other than the rat is consequently urgent. The relatively low content of TSH in the blood and hypophysis of the guinea pig (D'Angelo and Gordon, 1950) suggests a type of thyroid-pituitary balance which may respond markedly to proper endocrine manipulation. Accordingly, a detailed investigation of thyroid and pituitary parameters is being made in this form, one phase of which has already been reported (D'Angelo, Paschkis, Cantarow, Siegel, and Rivero-Fontan, 1951). In the present study, blood and pituitary TSH levels have been determined and correlated with morphologic changes in the thyroid and hypophysis of guinea pigs made hypothyroid with an anti-thyroid agent.

METHOD

A total of 110 normal and experimental guinea pigs were used. Animals received propylthiouracil (0.1%) in the basic ration (Purina Omolene) for periods of 7, 40, and 80

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days, with control animals receiving the basic ration alone. At sacrifice, the thyroid and hypophysis were quickly removed, weighed on a microtorsion balance, and prepared for microscopic study. Pituitaries were divided sagittally, with portions fixed either in Helly's of Champy's fluid. Tissue from the latter were post-osmicated (4–5 days at 37° C.) to demonstrate the Golgi apparatus. Serial sections of paraffin-embedded pituitaries were cut at 2, 3 and 4 micra, and were stained with modified Masson or azan procedures. Thyroid response was evaluated by microhistometric measurement of the acinar cell height (a total of 200 follicular cells per thyroid lobe was measured). Blood for the bioassay study was obtained by cardiac puncture under light ether anesthesia. The sera of animals in respective control and experimental groups were pooled and frozen until ready for assay by the stasis tadpole technic (D'Angelo and Gordon, 1950). The pituitary extract was prepared by a standardized procedure used in the laboratory. Seven anterior lobes (posterior lobe removed by blunt dissection) were homogenized in acidified saline (0.25% glacial acetic acid). The material was extracted twice and, after centrifugation, the clear supernatant fluid was bioassayed for TSH in the tadpoles.[1] Extract volumes were adjusted so that each test animal received an equivalent of 1.4 milligrams of anterior pituitary tissue over a period of 4 injections. For blood assay a total of 0.30 cc. of serum was given in 6 injections.

TABLE 1. BLOOD THYROID-TSH BALANCE AND TSH CONTENT OF THE ANTERIOR PITUITARY IN THE GOITROUS GUINEA PIG

Mean body wgt.-gm. (no. pigs)	Days on drug	M. thyroid wgt.-mg. / 100 gm. body wgt.	M. thyroid cell hgt. (μ)	M. ant. pit.-mg. / 100 gm. body wgt.	Ant. pit. assay: Thy. cell hgt. (μ)	Ant. pit. assay: Est. TSH in extract	Blood assay: H-limb increase (mm.)	Blood assay: thyroid cell hgt. (μ)
558±75[1] (7)	0	15.1±1.6	7.1±0.2	1.6±0.07	7.8±0.3	1.6μg.[2] (0.8–2.4)	2.4±0.1[3]	5.1±0.1[3]
435±50 (7)	7	17.2±0.9	7.5±0.3	1.8±0.15	6.9±0.2	0.4μg. (0.2–0.3)	2.4±0.1	5.9±0.1
384±20 (7)	40	26.8±2.6	12.0±0.9	1.9±0.12	8.6±0.3	4.0μg. (2.5–6.0)	1.8±0.1	5.8±0.1
486±50 (7)	78	68.9±9.5	12.2±0.3	2.2±0.09	9.5±0.2	14.0μg. (10–20)	1.2±0.1	5.9±0.2
357±30 (7)	0	16.1±1.0	7.1±0.3	1.8±0.08	7.6±0.2	1.2μg. (0.5–1.6)	2.6±0.2	5.3±0.1

[1] Mean body wgt. at sacrifice ± avg. dev.; in all other columns ± values represent standard error of mean.
[2] TSH content of guinea pig pituitary extracts given in mean microgram equivalents (range) of a previously standardized thyrotropin preparation (D'Angelo, Endocrinology 48: 249, 1951).
[3] Refers to mean hind-limb increase and thyroid cell height in stasis tadpoles receiving guinea pig sera.

RESULTS

The summarized data (Table 1) confirm the earlier finding (D'Angelo *et al.*, 1951) that there is appreciable delay in the onset of morphologic change in the thyroid. Neither thyroid weight nor acinar cell height was changed from normal with seven days treatment of propylthiouracil. The TSH content of pituitary and serum was significantly altered, however. TSH concentration in the adenohypophysis decreased to one-fourth of normal, whereas, blood TSH, not detectable in normal guinea pig serum,

[1] Grateful acknowledgment is made to Mrs. Ada Schafer and Mrs. Betsey Brownlee for technical assistance.

rose to levels approximating 0.001 JSU[2]/ml. With continued treatment, elevation of the thyroid cell height, colloid release, and hyperplasia occurred (Fig. 1). The anterior pituitary eventually enlarged, and TSH concentrations in the gland were markedly increased (approximately 280% and 1000% of normal at 40 and 78 days respectively). The elevated blood TSH titers were maintained at a relatively constant level throughout. The decreased hindlimb extension in tadpoles given such sera indicated that the heightened TSH titers were accompanied by simultaneously lowered levels of thyroid hormone. This shift in the thyroid-TSH blood equilibrium follows the inhibition of thyroid hormone formation with goitrogen (D'Angelo and Gordon, 1950).

The thyrotrophic hormone shifts in blood and hypophysis, as well as the morphologic changes in the thyroid, appeared to be closely dependent upon alterations in the pituitary basophiles. (The detailed changes in hypophysial cell types, as related to disturbances in the thyroid-pituitary axis, will be considered elsewhere; only the major changes which occur with goitrogen will be given here.) The basophilic population, normally concentrated around the area of the stalk along the median, ventral, aspect (bone surface) of the gland showed definite structural changes as early as several days following propylthiouracil treatment. These involved a decrease in the numbers of large, finely granular, deeply staining basophiles and a simultaneous increase in small, coarsely granular cells with hyperchromatic nuclei (Figs. 2 and 3). After prolonged treatment—when TSH concentrations in the gland were appreciably augmented—there was marked hypertrophy and hyperplasia of both light and deeply staining varieties of basophiles, their distribution in the gland becoming widespread (Fig. 4). The dark, irregularly-shaped basophiles were unusually large. These cells were further characterized by a fine cytoplasmic granulation, hypertrophied Golgi networks, and by large nuclei bearing numerous prominent nucleoli (Figs. 5 and 6). Vacuolation was rare, however, and no obvious counterpart to the so-called "thyroidectomy" or "goiter" cell of the rat appeared. There did appear after prolonged treatment, however, certain rather large basophilic cells in the stalk area displaying a coarsely punctated granulation in an otherwise clear chromophobic cytoplasm. Although the acidophiles remained numerous throughout treatment, an appreciable proportion of these cells showed regressive changes. Cytoplasm was visible only as rims or crescents of sparsely granular acidophilic material. Nuclei were pycnotic and the Golgi appratus became inconspicuous. Many acidophiles retained the normal structural pattern, however.

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[2] Junkmann-Schoeller Units.

DISCUSSION

The initial diminution and subsequent marked rise in pituitary TSH content found with chronic goitrogen treatment indicates the necessity of considering the duration factor in cyto-physiologic studies of the adenohypophysis. Failure to consider this may well account for the sharply conflicting reports regarding TSH content of the rat pituitary in thyroxine deficiency (Griesbach and Purves, 1943; Turner and Cupps, 1940; Gordon *et al.*, 1945). The importance of accurate definition of blood TSH levels in conjunction with pituitary examination is equally obvious. It can be safely inferred from the present study that the inhibition of thyroid hormone formation with propylthiouracil elicits augmented release of TSH from the pituitary. With short periods of treatment, thyrotrophic hormone release into the circulation must occur before the mechanism of production is appreciably activated. As thyroid hormone deficiency continues, TSH production eventually surpasses release, so that hormone stores in the pituitary now augment. The relatively constant blood levels of the hormone throughout may mean that delivery of TSH into the circulation, under these conditions of thyroid hormone deficiency, is at a maximal rate. The possibility still exists, however, that increased production of TSH in the pituitary is accompanied by an increased delivery rate of the hormone into the blood with this augmented release of TSH counterbalanced, in turn, by excessive destruction or inactivation in the circulation.

The strong basophilia of the pituitary found at a time when TSH content of blood and hypophysis is high makes it more than probable that the genesis of TSH resides in this cell type. The persistence of the acidophiles in the hypophysis of the goitrous guinea pig, as contrasted with their disappearance in the rat, does not complicate interpretation of the cytologic picture. Neither in numbers, granule content, nor in the behavior of the cytoplasmic organoids, can the acidophiles be correlated with change in TSH stores of the pituitary. The association of this cell type with continued

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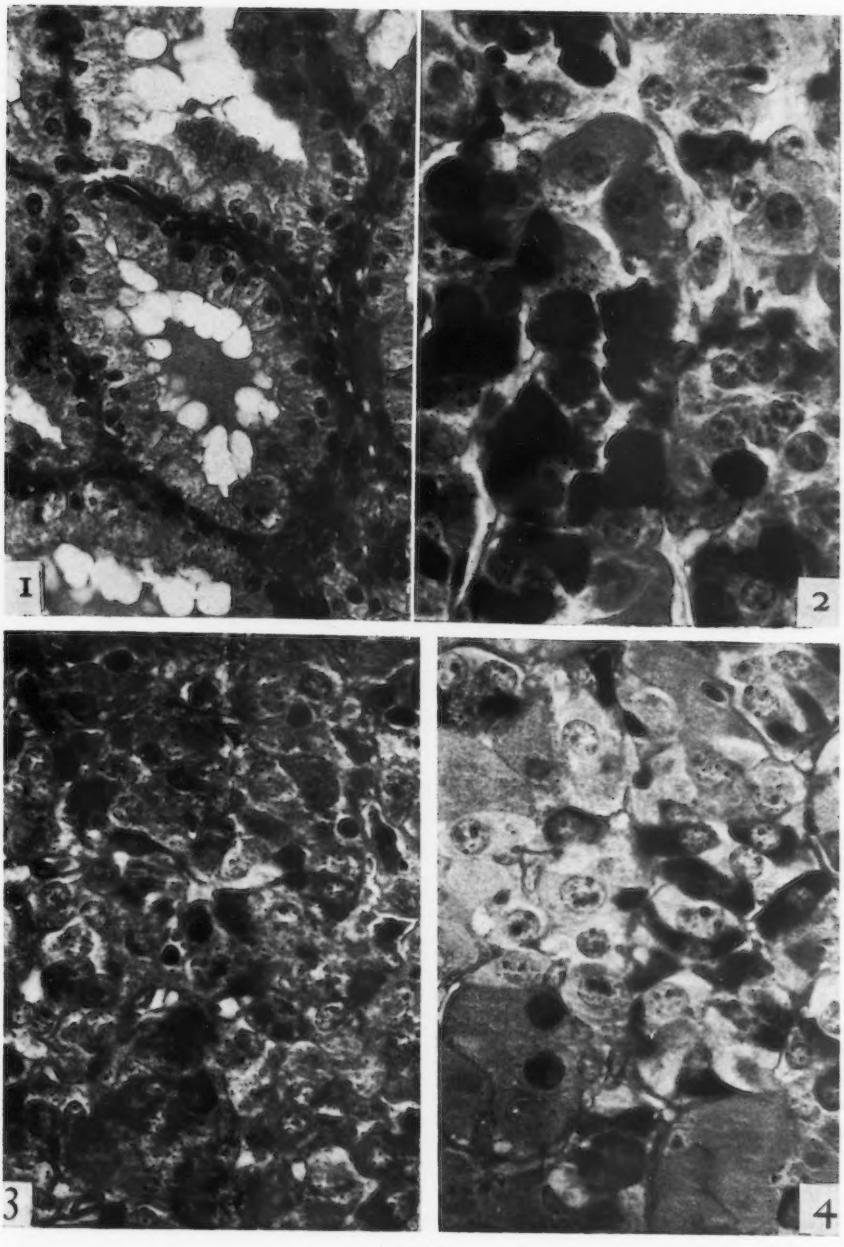
EXPLANATION OF FIGS. 1–4

FIG. 1. The histologic response of the thyroid gland after 78 days treatment with goitrogen (Bouin's Picro-Formol; H. and E., ×350).

FIG. 2. A section through the normal adenohypophysis displaying several large basophiles (upper right) and the more numerous smaller acidophiles (black, elongate elements at left; Helly's, Masson, ×700).

FIG. 3. The widespread degranulation and diminution in size of the chromophiles in the pituitary after 5 days on drug (Helly's, Masson, ×700).

FIG. 4. The adenohypophysis of a guinea pig after 85 days on goitrogen. The basophiles (grey) are hyperplastic; acidophiles (black) are small with rims or crescents of granular cytoplasm (Helly's, Masson, ×700).



FIGS. 1–4

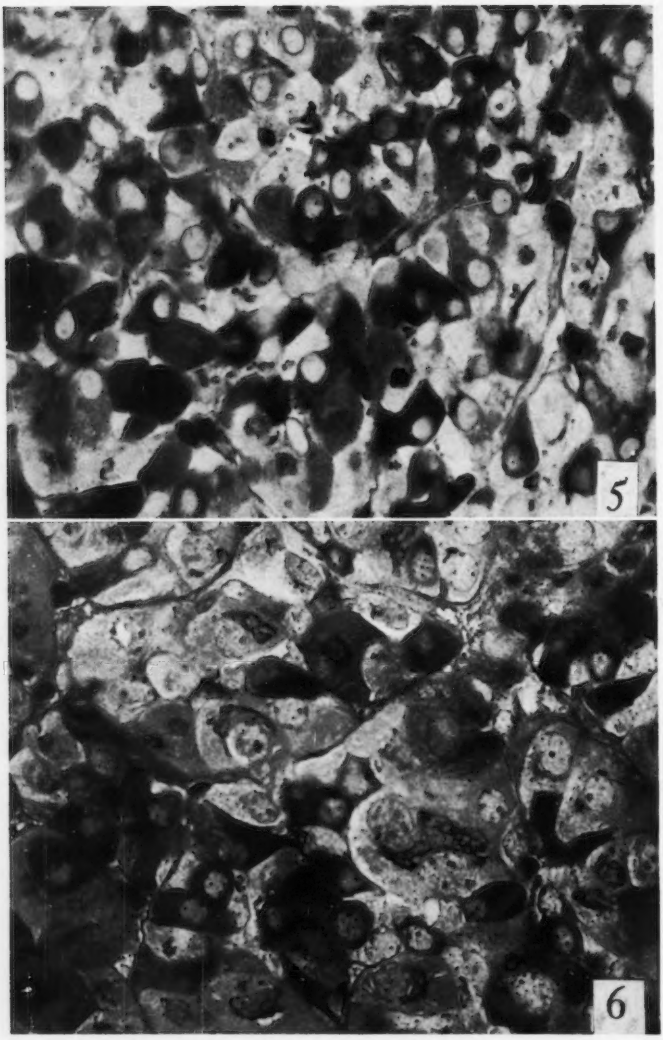


FIG. 5. A representative section of the normal anterior pituitary showing the relative size and configuration of the Golgi apparatus (black network) in the basophiles (light grey) and acidophiles (dark grey). In both cell types, the ovoid nuclei appear hyaline (Nassonov-Kolatschew, azo-carmine-aniline blue, ×700).

FIG. 6. A section comparable to that in Fig. 5 displaying the marked hypertrophy of the Golgi material in the basophiles after 78 days of propylthiouracil treatment. The Golgi apparatus is either normal or reduced in size in acidophiles. (Nassonov-Kolatschew, azo-carmine-aniline blue, ×700).

growth in the goitrous guinea pig is, on the other hand, more impressive. The absence of "thyroidectomy" or "goiter" cells in the guinea pig hypophysis recalls other major morphologic differences from the rat (e.g., the lack of "castration" cells in the guinea pig, Kirkman, 1937). Neither the morphology nor the distribution of the guinea pig basophiles suggests a basis for their separation into the beta and delta cells of Halmi (1950) or into the "thyrotrophs" and "gonadotrophs" of Purves and Griesbach (1951). Histochemical technics may do so, however, and these are now being applied to the guinea pig pituitary.

SUMMARY

TSH assays of blood and pituitary in hypothyroid guinea pigs disclose marked shifts in the hormonal balance. Periods of goitrogen treatment too short to effect appreciable anatomic change in the thyroid result in decreased pituitary TSH with elevated blood hormone levels. Prolonged treatment induces thyroid hyperplasia, pituitary enlargement, and marked increases in TSH concentration of the hypophysis. Blood TSH levels, though elevated, do not progressively increase. These hormonal shifts closely correlate with change in the pituitary basophiles. When TSH stores are depleted, the basophiles are decreased in size through loss of their specific granulation. Contrariwise, augmented TSH content of the adenohypophysis is associated with an increase in the size, numbers, and cytologic activity of the basophilic cells. Acidophiles remain numerous throughout; but a substantial number of these display regressive changes.

The results indicate that the source of thyrotrophin is in the basophiles, and that augmented production of the hormone by this cell type appears necessary to sustain elevated blood TSH levels essential for thyroid hyperplasia.

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EFFECT OF HYPOPHYSECTOMY ON I^{131} METABOLISM IN NORMAL AND THIOURACIL TREATED C_3H MICE

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INTRODUCTION

MANY studies have been made of the effect of hypophysectomy on the radioiodine metabolism of the rat (Leblond, Sue and Chamorro, 1940; Leblond and Sue, 1941; Morton, Perlman, Anderson and Chaikoff, 1942; Cortell and Rawson, 1944; Chaikoff and Taurog, 1949; VanderLaan and Greer, 1950; Ghosh, Woodbury and Sayers, 1951; Randall and Albert, 1951; Randall, Lorenz and Albert, 1951; Albert, Tenney and Lorenz, 1952). Similar studies in the mouse have not been reported as yet.

This paper presents the experimental findings made in a study of some effects of hypophysectomy on I^{131} metabolism in C_3H mice with either normal or hyperplastic thyroid glands.[1] Data are reported on the I^{131} uptake by the thyroid gland, on the percent of thyroid I^{131} in thyroxine, and on the concentration of radioiodide in the thyroid gland compared to that in the serum. It was found that the I^{131} content of the thyroid gland 25 hours after I^{131} injection undergoes an extensive and progressive decrease with increasing time intervals after hypophysectomy. In contrast, while the ratio of radiodide content of the thyroid gland to that of serum decreases after hypophysectomy, it remains elevated above 50.

METHODS AND MATERIALS

The radioiodine studies were made in male C_3H mice between four and five months of age. The mice were supplied by the Animal Production Unit of the National Institutes of Health.

Diet: Two groups of mice were studied. One group was fed from weaning a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner, Morris, 1952). After weaning, the other group was fed for three months a meal diet similar in composition to the stock diet but containing a supplement of 2.5 gm. of thiouracil per kilogram. It was then fed the stock diet without supplement for the remainder of the experiment.

Operative procedures: Hypophysectomy was performed through a parapharyngeal approach (Korteweg and Thomas, 1939) when the mice were about four months old. In the sham operation, the hypophysectomy procedure was followed up to and including

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[1] A preliminary report of these results was presented at the New York meeting of the Federated Societies in April 1952. (Wollman and Scow, 1952.)

the removal of the outer table of the occipital bone. Only those hypophysectomized mice are included in this report in which complete extirpation of the gland had been achieved as ascertained by visual examination (9×magnification) of the sella turcica and adjacent tissues. Serial sections of the sella turcica and adjacent bone, stained with hemotoxylin and eosin, were made of the hypophysectomized mice in certain groups as evidence of completeness of the operation. Testes weights were obtained in certain groups of normal and hypophysectomized mice.

I^{131} measurements: The I^{131} in thyroid glands or in extracts of thyroid glands was measured by gamma counting (Wollman, Morris and Green, 1951). The I^{131} in serum was measured by adding 0.05 ml. serum to 0.95 ml. of H_2O containing a mg. of carrier NaI. An aliquot of this solution was dried on a copper planchet and counted with an end window counter. The statistical counting error was less than three per cent by either method. The counting rate of a sample using the beta counter was 150 times that using the gamma counter.

Uptake 25 hours after I^{131} injection: In the 25-hour uptake studies, each mouse received intraperitoneally 10 μc. of carrier free I^{131} [2] and was killed with ether 25 hours later. The thyroid gland, after weighing, was placed in 1 ml. 2 N NaOH and its I^{131} content was determined. The thyroid gland of each mouse fed the thiouracil-free diet was then hydrolyzed on a steam bath, and the thyroxine fraction was isolated by the butanol extraction method of Taurog and Chaikoff (1946) and its I^{131} content determined.

Uptake at 1½ hours after I^{131} injection: One and one-half hours after receiving 10 μc. of I^{131} as described earlier, each mouse was anaesthetized with ether and bled from the heart. The thyroid gland was removed immediately thereafter and was placed in a glass homogenizer with 3 ml. of ice cold 8 per cent trichloroacetic acid solution containing a few mg. of NaI as carrier. It was maintained at a temperature below 5° C. for the remainder of the procedure. The I^{131} content was determined and the tissue was then homogenized in 8 ml. of the acid, centrifuged and the I^{131} content of the supernatant was measured. The serum I^{131} was determined. Test showed that a negligible fraction of the serum I^{131} was protein bound at this time.

The thyroid iodide/serum iodide concentration ratio: Each mouse in this series was given a subcutaneous injection of 0.5 ml. of freshly prepared solution containing 5 mg. propylthiouracil per ml. of 0.9 per cent NaCl which contained NaOH at a concentration of 0.04 normal. One hour later 10 μc. I^{131} was given intraperitoneally. One to two hours after I^{131} injection the mouse was anaesthetized with ether, a blood sample taken from the heart, and the thyroid gland was immediately removed and weighed. The I^{131} content of thyroid gland and serum were measured.

RESULTS

There was a body weight loss which varied from 4 to 6 grams in hypophysectomized mice (mean body weight at surgery was 30 gm.) during the first 4 to 7 postoperative days after which the weight remained unchanged. The three gram weight loss in the shams was transient; their body weights were back to preoperative values in 7 days. In the mice fed the stock diet, the thyroid weight, which was 2 mg. in normals, was decreased by 26 per cent at 7 days after hypophysectomy and by 39 per cent at 12 days. In

[2] The I^{131} used in this investigation was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

mice fed the thiouracil diet the thyroid glands averaged 19 mg. The thyroid weights of mice fed the thiouracil diet decreased by 15 per cent during the 13 days following hypophysectomy. However, the thyroid weights of the sham-operated controls decreased sufficiently so that the thyroid weights in the experimental and control groups never differed significantly. The mean and the standard deviation of the weights of a single testis in mice fed the thiouracil supplement was 86 ± 7 mg. in the shams, whereas in

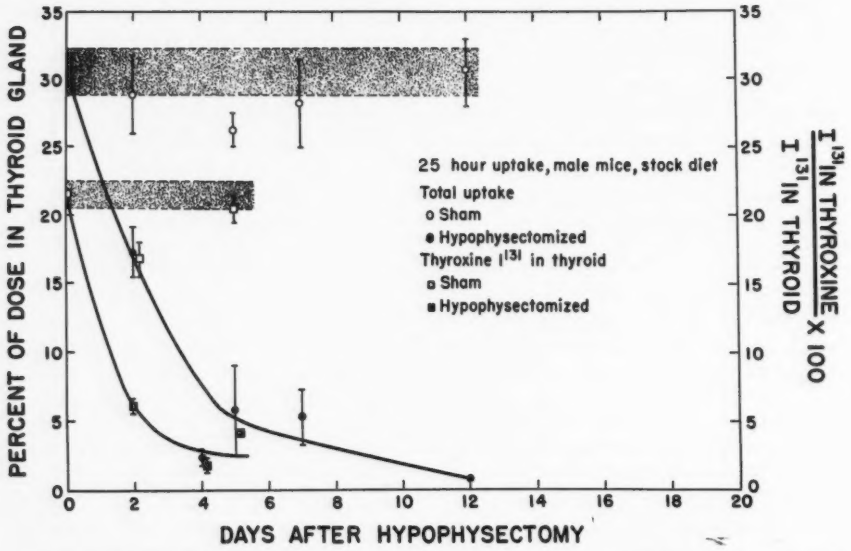

FIG. 1. Effect of hypophysectomy on I^{131} uptake and on percentage of thyroid I^{131} present in thyroxine fraction 25 hours after radioiodide administration in C_3H mice fed the stock diet. The shaded areas indicate the standard error range for the normals; the upper is for I^{131} uptake, the lower for thyroxine fraction. The vertical bar at each point represents the standard error. Four to six mice were used per point. The point for five days after hypophysectomy includes observations on only two mice.

hypophysectomized mice it was 77 ± 7 mg. at six days after hypophysectomy and 40 ± 11 mg. at 13 days.

The 25-hour I^{131} uptake at various time intervals after hypophysectomy in mice fed the stock diet is shown in Figure 1. There was a progressive decrease in the uptake of the thyroid glands in hypophysectomized mice during the postoperative period, with the uptake decreasing to 50 per cent of the control value by the second postoperative day.[3] The sham-operative

[3] In a few data collected on female mice the I^{131} uptake was 30 per cent higher than in corresponding male mice, but there was no significant sex difference in the I^{131} uptake in mice that had been hypophysectomized seven days.

procedure had no significant effect on the uptake even though the left lobe of the thyroid gland had been compressed during the operative procedure.

The per cent of thyroid I^{131} in the thyroxine fraction in these mice is also presented in Figure 1. By the second postoperative day, a marked reduction of the per cent of thyroid I^{131} in the thyroxine fraction to one-third of that for the shams was observed in hypophysectomized mice, there being a further decrease thereafter.

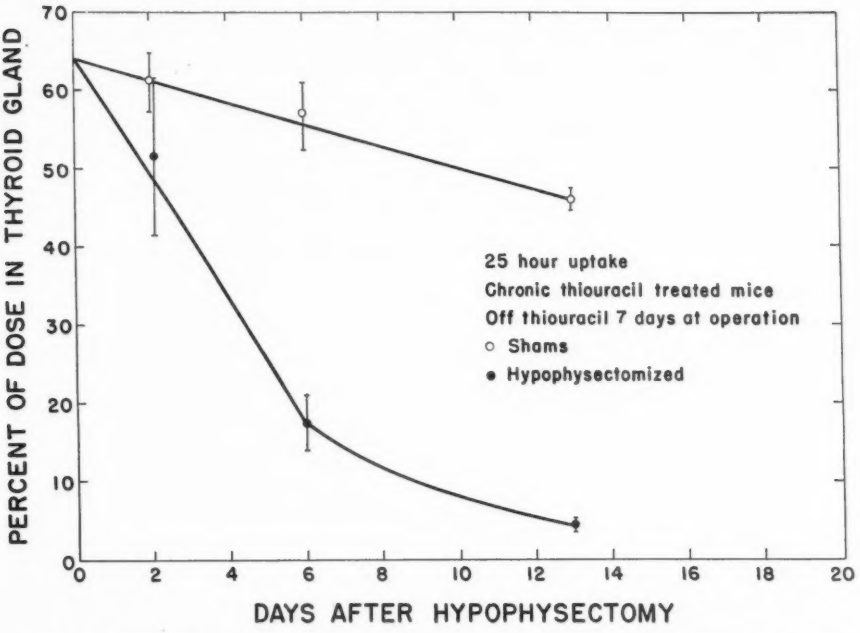

FIG. 2. Effect of hypophysectomy on uptake of radioiodide 25 hours after administration in C_3H mice fed 0.25% thiouracil diet up to seventh preoperative day and then fed the stock diet. Approximately 5 hypophysectomized mice and 6 sham operated mice were studied at each time interval.

The 25-hour I^{131} uptake in hypophysectomized and sham-operated mice fed the thiouracil diet is presented in Figure 2. The mice were fed the stock diet for seven days just prior to operation. Completeness of hypophysectomy was verified by examination of serial sections of the region of the sella turcica in those mice studied at 13 days after hypophysectomy. There was a progressive decrease in the I^{131} uptake following hypophysectomy in these animals, as observed also in those fed the stock diet. The gradual decrease in I^{131} uptake with increase in the postoperative time intervals ob-

served in sham-operated mice probably was the result of the withdrawal of thiouracil. The amount of I^{131} incorporated into the thyroxine fraction in these hypertrophied thyroids was not determined since preliminary studies had shown that it was too small to be measured accurately.

The effect of hypophysectomy on I^{131} uptake 1.5 hours after I^{131} injection in mice fed the thiouracil diet is shown in Table 1. The mice were fed stock diet for 18 days before operation. Completeness of hypophysectomy was verified in each experimental animal by examination of serial sections of the region of the sella turcica. This study was made to check the possibility that the small effect of hypophysectomy on 25-hour uptake at short time intervals after operation in animals fed thiouracil might be due in part to the fact that the maximum uptake in the thyroid glands of the controls occurs only a few hours after I^{131} injection, while the uptake in the

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON I^{131} IN THE THYROID GLAND AND SERUM IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS

		Hypophysectomized	Sham-operated
Number of mice		5	5
Thyroid I^{131}			
Total	counts/sec./100 mg.[1]	109 ±6	495 ±29
TCA-soluble	counts/sec./100 mg.	66.5 ±6	41.2 ± 5
Per cent TCA-soluble		61 ±4	8.3 ± 0.3
Per cent of injected dose			
Total		2.92 ±0.36	16.6 ± 1.5
TCA-precipitable		1.11 ±0.12	15.3 ± 1.5
Serum I^{131}	counts/sec./100 mg.[1]	4.11 ±0.26	2.62 ± 0.06
Thyroid iodide conc. / Serum iodide conc.		14.5 ±1.2	16.2 ± 1.9

[1] All counting rates reported as if measured by gamma counting. The serum data were obtained by beta counting, and were converted to the equivalent gamma counting data using an experimentally determined conversion factor.

Mice fed diet containing thiouracil for three months, fed stock diet 18 days, and then operated on. Studies were made on sixth postoperative day. Samples were taken one and one-half hours after I^{131} administration.

hypophysectomized animals increases more slowly, but continues to increase for a much longer time after injection. At this 1.5 hour time interval the total I^{131} in the thyroid glands of the hypophysectomized mice was only 20 per cent that of the controls, the difference being almost twice as great as that observed at 25 hours after I^{131} injection. The concentration of acid-soluble I^{131} in thyroid glands and the concentration of serum I^{131} are approximately 50% higher in the hypophysectomized mice than in the controls. As a consequence, the ratio of the concentration of acid-soluble I^{131} in the thyroid to serum radioiodide concentration was the same for both

groups. Acid-soluble I^{131} amounted to 60% of the total I^{131} in the thyroids of the hypophysectomized mice, but only 8% in the controls.[4] The amount of organic bound I^{131} in the thyroids of hypophysectomized mice was much less than in those of the controls.

The effect of hypophysectomy on the thyroid iodide/serum iodide concentration ratio in mice fed thiouracil is presented in Table 2. A statistically significant though small depression of this ratio as the result of hypophysectomy was observed only at thirteen days after operation, the latest time interval studied. The hypophysectomies in this last group, studied 13 days after operation, were shown to be complete by examination of serial sections of the region of the sella turcica. Two and one-half mg. of propylthiouracil was shown to be effective in blocking protein binding of radioiodide by the thyroid gland for at least five hours in these mice. The values of the ratios in the sham-operated mice were abnormally low, since mice fed either a goitrogen-free diet or thiouracil in this laboratory ordinarily have ratios of over 200. The low values observed may have been due to a somewhat higher iodide concentration in the particular batch of stock diet fed the mice in the time interval during which these studies were made.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON THYROID IODINE/SERUM IODIDE CONCENTRATION RATIO[1] IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS[2]

Days after operation	Concentration ratio	
	Hypophysectomized	Sham-operated
2	75 ± 8 (5)[3]	96 ± 4 (7)
6	90 ± 10 (6)	91 ± 9 (7)
13	67 ± 6 (6)	108 ± 9 (6)

[1] In order to prevent organic binding of radioiodide, each mouse was given a single subcutaneous injection of a solution containing 2.5 mg. of propylthiouracil one hour prior to injection of 10 μc of I^{131}. Samples were taken one to two hours after the I^{131} injection.

[2] Fed thiouracil diet three months, hypophysectomized after feeding stock diet seven days.

[3] Mean ± standard error; parenthesis encloses the number of animals examined.

DISCUSSION

The effects of hypophysectomy on the I^{131} metabolism in the mouse appear qualitatively similar to those in the rat. The results, however, suggest some interpretations of the effects of hypophysectomy which are different from interpretations in papers on the rat.

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration decreased progressively with increasing time intervals after hypophysectomy in mice fed either the stock diet or the thiouracil-con-

[4] Albert and Lorenz (1951) have obtained similar results in the rat.

taining diet. Although not shown in figure 1 the logarithm of the uptake of the thyroid glands decreased linearly with the time after hypophysectomy in the mice fed the stock diet. This indicates a constant fractional rate of decrease of the 25-hour uptake with time after hypophysectomy. This interpretation may be contrasted with that suggested by Randall and Albert (1951) who concluded that the thyroid activity in the rat reached a minimum of 10% of normal by six days after hypophysectomy (using maximum uptake of injected I^{131} as a measure of thyroid activity). The interpretation of these data on the rat as well as the present 25-hour uptake data is complicated by the fact that the maximum uptake reflects not only thyroid function but also kidney function, and in particular the fact that radioiodide excretion by the kidney is grossly decreased in the hypophysectomized animal (Albert, Tenney and Lorenz, 1952). This would tend to make injected I^{131} available to the thyroid gland in the hypophysectomized animal for a longer time than in the normal, and tend to decrease the apparent loss of thyroid activity if a long time interval between injection and thyroid I^{131} measurement is selected. For a reliable measure of thyroid activity it is important to study a characteristic which is relatively independent of kidney activity such as thyroid clearance or uptake at times so soon after I^{131} administration that only a small fraction of administered I^{131} could be excreted by the kidney even in the normal animals.

It is important to ascertain which of the factors involved in the accumulation of I^{131} by the thyroid gland are grossly affected after hypophysectomy and responsible for the observed decreased accumulation. The preliminary observations on organic bound I^{131} six days after hypophysectomy in the mice with hyperplastic thyroid glands indicates a marked decrease in organic bound I^{131} in the hypophysectomized mice. This correlates well with the prompt decrease in the proportion of thyroid I^{131} found in the thyroxine fraction in mice fed the stock diet and previously reported for the rat (Morton, Perlman, Anderson and Chaikoff, 1942). Both organic binding of I^{131} and formation of thyroxine are presumably linked to oxidative processes in the cell.

The decrease in the organic bound I^{131} in the thyroid glands of the hypophysectomized mice with hyperplastic glands does not appear to be due to a lack of radioiodide in the gland since the acid-soluble I^{131} was actually higher in their glands than in those of the control mice. If the relationships at 1.5 hours after I^{131} injection are representative of those at earlier times it would suggest that the process of organic binding of I^{131} was grossly impaired in hypophysectomized mice.

The values reported for thyroid iodide/serum iodide concentration ratios of mice hypophysectomized after chronic feeding of thiouracil are almost always above 50. Many additional measurements of this ratio in mice hypophysectomized after feeding a thiouracil-free diet since weaning,

to be reported elsewhere, have repeatedly confirmed the above observations. These results in the mouse differ markedly from those in the hypophysectomized rat reported by Greer (1949) who found that the ratio approaches approximately 7, and by VanderLaan and Greer (1950) who found that the ratio frequently approaches 1. The relative insensitivity of this ratio to hypophysectomy in the mouse makes it possible to recognize the importance of the binding process as an important factor in the decreased accumulation of I^{131} in hypophysectomized mice.

SUMMARY

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration in male C_3H mice with normal or hyperplastic thyroid glands decreased progressively with increasing time after hypophysectomy. The proportion of the thyroid I^{131} in the thyroxine fraction also decreased rapidly after hypophysectomy in mice fed the stock diet. The thyroid iodide/serum iodide concentration ratio in the mice with hyperplastic thyroids exhibited a decrease during the 13 days after hypophysectomy but remained elevated above 50.

The decreased accumulation of bound I^{131} in the thyroid glands of these hypophysectomized mice did not appear to be due to a decrease in thyroid radioiodide concentration.

Acknowledgement

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MECHANISMS OF PARTIAL BLOCKADE OF THE STRESS RESPONSE IN RATS BY DIBENAMINE ANALOGUES[1]

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INTRODUCTION

IT IS generally agreed, following the pioneer work of Long and Fry (1945) and Vogt (1945), that physiological dosages of epinephrine will activate the release of adrenocorticotrophin (ACTH) from the adenohypophysis. However, there is considerably less accord as to the mechanism by which epinephrine exerts this effect. Long and his colleagues (Gershberg *et al.*, 1950; McDermott *et al.*, 1950) have presented strong evidence, confirmed by Fortier (1951 b), that injected epinephrine may act directly on the adenohypophysis. Hume and collaborators (Recant *et al.*, 1950) suggest that epinephrine activates the anterior hypothalamus to liberate a humoral substance which, in turn, stimulates the hypophysis. Sayers (1950, 1951) considers that epinephrine increases tissue utilization of cortical steroids and that their depletion from the blood "fires" the ACTH release mechanism. Porter (1952) has recently reported direct evidence that epinephrine activates the posterior hypothalamus and that lesions in this region prevent stress-induced discharge of ACTH. DeGroot (1952) also favors the hypothalamus as the primary site of epinephrine action, for he found that epinephrine would not stimulate lymphopenia in mice with their hypophyseal portal systems damaged.

The hypothesis that reflex secretion of endogenous epinephrine is essential in the natural mechanism by which stress activates ACTH release is confronted by similar lack of agreement. Gordon (1950 a, b) reported a normal response to "alarming" stimuli in rats with demedullated adrenal glands. This was denied by Gershberg *et al.* (1950) and Dury (1950). The controversy as to whether epinephrine is a specific link in the pituitary-adrenal mechanism or whether it acts like other non-specific stresses, is treated in the discussions of recent symposia reviews by Pincus (1950), Sayers (1951), Harris (1952) and Long (1952). Long's group has proposed a

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dual mechanism in which systemic epinephrine is responsible for an initial *autonomic* phase of pituitary stimulation but not for the later *metabolic* phase.

A barrier to the acceptance of a specific role for intrinsic epinephrine is the almost universal failure of attempts with adrenergic blocking agents to interfere with ACTH release. Partial blockades of the mechanism by ergotamine (Ronzoni and Reichlin, 1950) and by Dibenamine (Seifter *et al.*, 1949; Paschkis *et al.*, 1950; Ronzoni and Reichlin, 1950) have been reported. Not only was the degree of blockade slight but there was no attempt made to test whether even the partial effect was actually due to adrenergic blocking properties of the drugs. This latter consideration assumes importance in view of results to be presented below. Moreover, in the hands of other investigators, Dibenamine (Tepperman and Bogardus, 1948; Fortier, 1951 a), ergotamine (Tepperman and Tepperman, 1950; Gershberg *et al.*, 1950) and dihydroergocornine (August and Gubner, 1949) have been completely ineffective in interrupting the stress response. Whereas these negative results are inconclusive, they have failed to lend support for a significant role of endogenous epinephrine in the ACTH release mechanism.

In 1950, Sawyer *et al.*, reported that a new adrenergic blocking agent SKF-501 (N [9-Fluorenyl]-N-ethyl-β-chlorethylamine hydrochloride) was more highly effective than Dibenamine in blocking the neurogenic stimulus for LH (ovulating hormone, gonadotrophin) release from the adenohypophysis of rabbits and rats. SKF-501 has been shown to have 3-10 times as potent adrenergic blocking properties as Dibenamine (Fellows, 1949; Sawyer *et al.*, 1950). In adrenergic-blocking dosages, SKF-501 prevented ovulation in 80 per cent of the rabbits and rats tested. The mechanism of blockade was apparently anti-adrenergic activity since the effectiveness of SKF-501 was not shared by 2-dibenzylaminoethanol (D-ol), a Dibenamine derivative affecting the central nervous system but lacking adrenergic-blocking capacity. It seemed desirable, therefore, to examine the effects of both SKF-501 and D-ol on the stress response. The present study reveals that SKF-501, but not D-ol, largely blocks the stress-response to injected epinephrine in rats as evidenced by effects on the eosinophil count. Interestingly enough, both SKF-501 and D-ol prevent the autonomic phase in response to such non-specific stresses as cold and formalin injection. The results are interpreted as indicating that D-ol interrupts the reflex arc for neurogenic secretion of endogenous epinephrine in response to non-specific stress, and that SKF-501 may function in this manner as well as having the capacity to block the action of epinephrine at its effector site. The results are consistent with the sequence proposed in the dual hypothesis of Long *et al.* (*op. cit.*). Part of the present results, with

tentative interpretations somewhat at variance with the present ones, have appeared in abstract form (Sawyer and Parkerson, 1951; Sawyer, 1952).

MATERIALS AND METHODS

The experiments fall into two principal groups: those conducted in North Carolina (Series A) and those in California (Series B). In Series A, male rats of the inbred Vanderbilt (Osborne-Mendel) strain, ranging in weight from 220–360 grams, were used in the hematological study. The rat room was air-conditioned at a summer temperature of about 27° C. The animals were kept in wire mesh cages, not more than three to a cage, and fed *ad lib* on Purina Dog Chow. Lighting conditions were controlled by a time switch to give 14 hours of light per day (Everett, 1948). In Series B, Wistar rats ranging in weight from 150–260 gm. were employed. Lighting and temperature conditions were not rigidly controlled but on the day of each experiment, care was taken that the rats not be disturbed prior to the administration of the controlled stressing stimulus. Other conditions were similar to those in Series A. In both series, the stressing stimulus was applied routinely between 9–10 A.M.

The method employed to assess ACTH release was the eosinophilic response of Thorn *et al.* (1948), and direct eosinophil counts were made with a modification of Randolph's (1944) phloxine-propylene-glycol diluent. A small blood sample was collected from the tail vein into an oxalated depression in a paraffin block. Within a few minutes, in Series A, three WBC pipettes were filled to the 0.1 mark and diluted to the 1.1 mark. They were shaken for about 30 seconds and set aside for at least 15 minutes for staining. After shaking again at a fairly rapid rate for 2 minutes in a mechanical shaker, each of two pipettes was used to fill 4 Spencer Bright-Line chambers, with a 30-second period of shaking between chambers. If the 36-square counts for each of the two pipettes checked within 15%, the third pipette was not used; if not, the average of all three was taken. Most of the Series A eosinophil counts for control rats ranged between 30–80 cells per cubic mm., and repeated counts on individual animals, with a few days interval between bleedings, gave very similar values. These counts are lower than most of those reported heretofore, indicating that strain differences in rat eosinophil levels may exist. Most of the Series B Wistar rat eosinophil counts ranged between 100–300 cells per cubic mm. Seventy-two squares filled from 2 WBC pipettes were counted in the eosinophil determinations and rats were not used whose pre-stimulation level was less than 70/cu. mm.

Three types of stimuli for ACTH release were employed: epinephrine, cold stress, and formalin. Epinephrine (Parke-Davis Adrenalin, 1/1000–1/5000) was injected subcutaneously at a dosage of 0.2 mg./kg. Ten per cent formalin was injected subcutaneously at a constant dosage of 0.3 ml. As a cold stress stimulus, rats were kept for one hour in a refrigerator room at a temperature of 8–10° C., nearly 20° C. less than the rat room temperature. In the cold stress groups, blood samples were taken at 0 and 4 hours, so the stimulus includes handling and bleeding as well as the temperature changes. Control blood samples in the Series A epinephrine group were taken a few days before or after the experimental 4-hour sample, so that the effects of epinephrine were not complicated by additional stress. The Series B eosinophil counts assayed blood samples taken at 0, 1 and 4 hours in both the epinephrine and formalin series, so the handling and bleeding element in the stress stimulus was considerable. Routinely there was a 4-day recovery period between consecutive stress experiments on a given animal, and more than a week was allowed for recovery from SKF-501 or D-ol injection.

Sodium pentobarbital (Nembutal, Abbott), which was employed intraperitoneally

in preliminary experiments in Series A, was in the form of the veterinary preparation in an alcohol-propylene-glycol-water vehicle or a 2 per cent solution in Ringer-Locke. The latter solution, which was made up just before use, was warmed to body temperature and injected intraperitoneally at a dosage of 30–50 mg./kg.

The adrenergic blocking agent, SKF-501, was dissolved in a propylene-glycol vehicle at a concentration of 50 mg./ml.; this was diluted for use to 5 mg./ml. with Ringer-Locke solution. Intravenous injections were performed into the lesser saphenous vein below the ankle as described earlier (Sawyer, Everett and Markee, 1949). The dosage adopted for use, 10 mg./kg., is above the MLD: in the present experiments 2 rats receiving this dose died during the following night. This dosage level was chosen because of its demonstrated ability in the rat and the rabbit to block the stimulus for release of pituitary LH without interfering with the release mechanism itself or with the action of hormone on its target organ (Sawyer *et al.*, 1950). Dibenzylaminoethanol was injected intravenously at a concentration of 10 mg./ml. in saline, and the dosage of 15–20 mg./kg. usually induced immediate convulsive muscular activity which lasted about one minute. Thereafter, the rats' motor activity appeared to be depressed.

RESULTS

Stress responses induced by Nembutal, SKF-501, and D-ol. Our first experiments were an unsuccessful attempt, with Nembutal anesthesia, to stabilize the eosinophil count against depression from handling and bleeding. It was hoped that even intravenous injections of SKF-501 might be made under anesthesia without inducing an alarm reaction. Recant *et al.* (1950) and Ronzoni (1950) had reported such a stabilizing effect of the anesthetic. As summarized in Figure 1, our results were completely negative in this respect. Nembutal itself, in confirmation of Ludewig and Chanutin (1947), acted as a stress stimulus leading to a 50% reduction in the eosinophil count after 4 hours. It appeared to make little difference whether the Nembutal was in alcohol-propylene-glycol-water or in Ringer-Locke buffered with sodium carbonate, at room temperature or warmed. Nor did "training" the rats with twice-daily mock injections for a week lessen the stress response.

In spite of the fact that its central excitatory effects are much less severe than those induced by Dibenamine (Sawyer *et al.* 1950), intravenous injection of 10 mg./kg. SKF-501 was followed by a marked reduction in circulating eosinophils (Fig. 1). When the SKF-501 injection was preceded by intraperitoneal Nembutal the decrease was somewhat less, but the number of cases was too small to make the difference significant. Even if it were significant and if SKF-501 were completely ineffective in blocking ACTH release, a 60 per cent decrease in eosinophils is already so great that one could not expect much further reduction in response to cold stress or epinephrine. Inasmuch as the adrenergic blocking capacity of β-chloroethylamine derivatives lasts for days (Nickerson, 1949) we next tested whether the eosinophil count would return to normal by 24 hours after

injection of SKF-501. This was observed to be true (Fig. 1), and the finding allowed us to examine the drug's capacity to block the eosinopenic response to stress at that time. Marked eosinopenia was also induced by injection of D-ol, followed within 24 hours by a return to nearly normal levels (Fig. 1). Later results with Wistar rats (Series B) revealed eosinophil counts characteristically higher than normal, 24 hours after injection of either SKF-501 or D-ol.

To demonstrate that adrenergic blockade by SKF-501 does indeed persist for 24 hours after treatment, we injected 3 rats with 5 mg./kg.

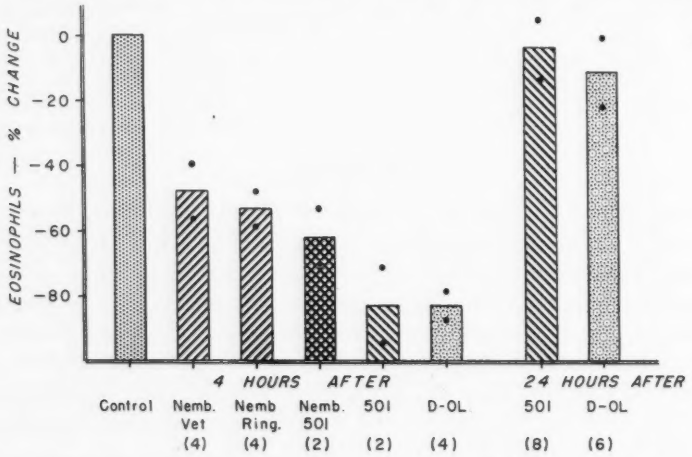

FIG. 1. Results of preliminary experiments in Series A. The effects of Nembutal, SKF-501, and D-ol on circulating eosinophils are expressed as percentages of the 0-hour counts. The figures in parentheses represent the number of rats employed in each group. Each set of dots limits the standard error of the mean.

SKF-501 (only one-half our usual dose) and followed at 24 hours with 0.2 mg./kg. intravenous epinephrine. This dosage of epinephrine was found in preliminary experiments to be in the realm of twice the LD_{100}, and in the present series it killed 2 control rats and 3 rats injected 24 hours earlier with 15 mg./kg. D-ol, in less than 5 minutes, all with symptoms of pulmonary edema. The 3 SKF-501-epinephrine-treated rats showed no such symptoms, and all survived. Thus SKF-501, at a dosage only half as great as that used in our ACTH experiments, protects at 24 hours against a twice-lethal dosage of epinephrine, whereas D-ol, at full dosage, lacks the capacity to block epinephrine effects.

Effects of SKF-501 and dibenzylaminoethanol on the 4-hour eosinophil responses to epinephrine and to cold stress. The results are summarized in Figure 2. Each of the columns represents data on at least 8 rats except the

last one in which only 2 animals were employed. However, only 17 rats were used in this whole series, and as far as possible the same rats were subjected to a given stressful stimulus and attempted blockade of that stimulus. It is apparent that either subcutaneous epinephrine or exposure to cold stimulates a marked decrease in the number of circulating eosinophils, about a 70 per cent depression in either category. The eosinopenia stimulated at 24 hours after SKF-501 injection, though still considerable in the cold-stressed or epinephrine-treated rats, is significantly reduced.

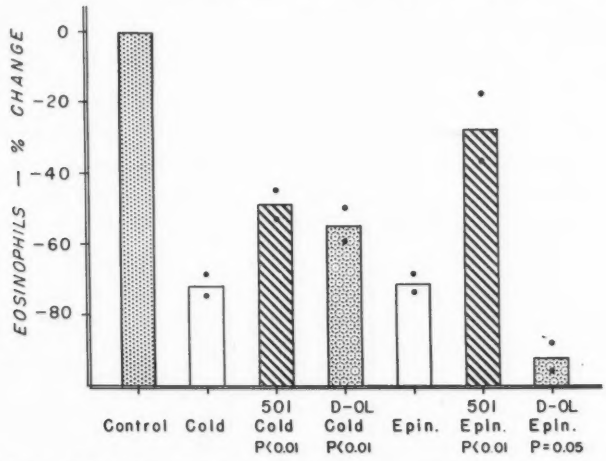

FIG. 2. Effects of "blocking" agents on the 4-hour eosinophil response to cold stress and to epinephrine (Series A). The P values for the differences between stressed and stressed-"blocked" animals were calculated from eosinophil-count data, not from the percentage-change columns shown in the figure.

$P < 0.01$ in either case between the eosinophil levels of SKF-501-treated and non-treated groups. The failure to respond 24 hours after SKF-501 is probably not attributable to the initial non-specific stress of SKF-501 itself, for 4 controls each gave a normal eosinophil response to epinephrine or cold stress 24 hours after epinephrine or cold stress, respectively. Interestingly enough, however, D-ol "blocked" the response to cold stress about as effectively as SKF-501 without preventing epinephrine-induced eosinopenia at all. The degree of eosinopenia in the D-ol-epinephrine group was, if anything, greater than in the controls lacking D-ol.

Effects of SKF-501 and dibenzylaminoethanol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin. These experiments were undertaken after the appearance of Long's dual hypothesis (McDermott *et al., op cit.*) to determine whether the autonomic phase of pituitary stimulation might be prevented by adrenergic blockade. The results

are summarized in Figure 3. Pre-treatment with SKF-501 but not with D-ol completely eliminated the eosinopenia at 1 hour after epinephrine. The rats pretreated with D-ol again showed a response to epinephrine, perhaps more marked than the controls. The 4-hour results confirm the

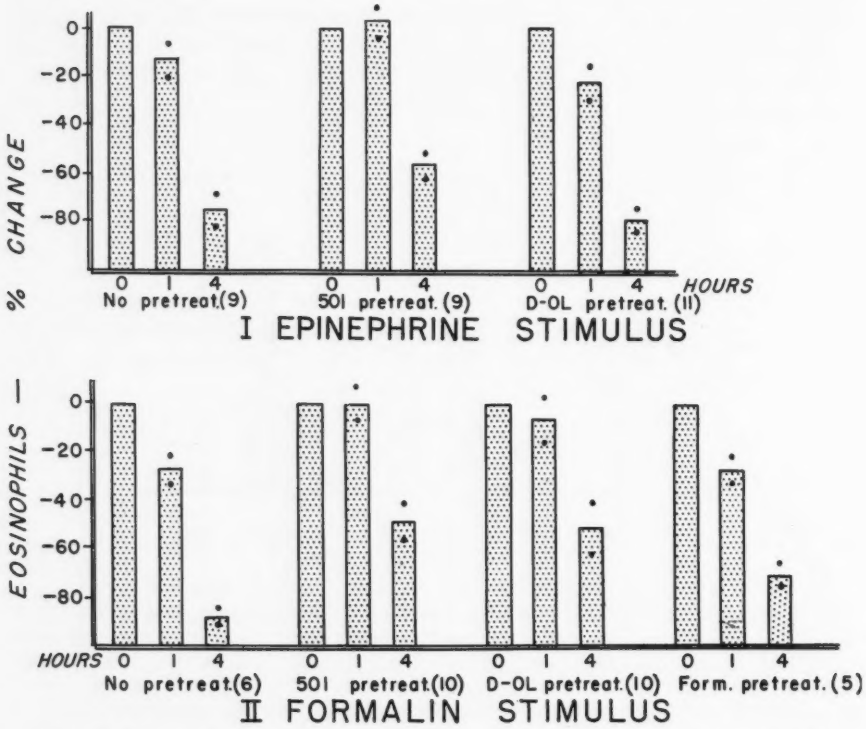

FIG. 3. Effects of pretreatment with SKF-501 and D-ol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin (Series B). In group I, epinephrine was injected subcutaneously, 24 hours after treatment with SKF-501 or D-ol. In group II, formalin was injected subcutaneously 24 hours after treatment with SKF-501, D-ol or formalin itself. Figures in parentheses represent numbers of rats employed in each group.

earlier findings with epinephrine, except that the SKF-501 column reflects the added stress of handling and bleeding at 0-hour and 1-hour collectively.

Formalin (Fig. 3) induced an eosinopenia at 1 hour at least as great as that stimulated by epinephrine and the effect was not lessened by formalin stress on the preceding day. However, both SKF-501 and D-ol appeared to prevent this 1-hour response to formalin and to diminish the 4-hour response in the same degree as they had lessened the response to cold.

DISCUSSION

It seems clear from the present results that β-chloroethylamine adrenergic blocking agents may partially prevent stress-induced eosinopenia by more than one mechanism. SKF-501 and dibenzylaminoethanol exert similar degrees of "blockade" against formalin or cold stress but differential effects against epinephrine. The blockade of formalin and cold stress by D-ol must be exerted by some means other than adrenolytic action. The fact that epinephrine is effective in stimulating a normal eosinopenia in spite of D-ol indicates that the final stages in the eosinopenic mechanism are not disturbed by chloroethylamine derivatives. Therefore, the failure of cold or formalin to induce a full-fledged eosinopenia after D-ol or SKF-501 cannot be attributed to the inability of the effector end of the eosinopenic mechanism to respond. Presumably the adrenal cortex and the pituitary ACTH release mechanism are intact.

While lacking the capacity to counteract epinephrine, D-ol blocks the autonomic phase of eosinopenia in response to cold or formalin. It seems most likely that it does so by interfering in some way with the stimulus for release of endogenous epinephrine. In this capacity its action may be analogous to those of barbiturates which were reported by Sayers and Sayers (1946) and Gellhorn (1949) to interfere with adrenal ascorbic acid depletion and lymphopenia, respectively, in the rat after generalized stress but not after injection of epinephrine. D-ol does, in fact, appear to be a depressant after a brief period of excitation of the central nervous system.

SKF-501 exerts a similar though less marked depressant effect, and its primary action against formalin and cold stress may be the same as that exerted by D-ol. However, SKF-501 has, in addition, very potent adrenolytic properties and these are credited with its blockade of epinephrine-induced eosinopenia. There is no evidence that epinephrine was responsible for any of the 4-hour eosinopenia encountered after cold, formalin or epinephrine injection in SKF-501-treated animals. Where non-specific stress was least (i.e. epinephrine injection without bleeding), the 4-hour eosinopenia was least evident.

It is significant that SKF-501 does not prevent the 4-hour eosinopenia from cold or formalin any more effectively than does D-ol. This must mean that under either SKF-501 or D-ol, neither cold nor formalin stimulates a systemic discharge of epinephrine and that the eosinopenia which is achieved is effected via ACTH release from another mechanism, e.g., the "metabolic" phase. The results allow one to make a rough calculation of the relative part played by epinephrine in both autonomic and metabolic phases of eosinopenia. With epinephrine eliminated via blockade, the non-specific metabolic effects induced about zero % and 50% eosinopenia at 1 and 4 hours, respectively, whereas the combined autonomic and meta-

bolic phases registered about 20% and 70% eosinopenia after 1 and 4 hours. These crude calculations indicate that epinephrine is responsible for a 20% eosinopenia which appears to be additive at 4 hours to the 50% metabolic effect.

The extreme eosinopenia following injection of SKF-501 itself may well involve both autonomic and metabolic phases, since certain adrenergic blocking properties of the β-chloroethylamine agents take several minutes to develop (Nickerson); probably too long to allow SKF-501 to block its own autonomic phase. Apparently neither SKF-501 nor D-ol exerts central depressant effects rapidly enough to prevent its own stressing stimulus from releasing endogenous epinephrine.

Our results, while emphasizing that the locus of epinephrine action is fundamentally different from that of non-specific stressing agents, provide only very indirect evidence as to the actual site or sites of adrenergic activity. Subcutaneous epinephrine must exert a systemic effect beyond local stimulation of sensory nerve endings; for if it did not, the central depressant effects of D-ol ought to block it as effectively as cold or formalin. If D-ol and the barbiturates (Sayers and Sayers, 1948; Gellhorn, 1949) exert their blockade of the generalized stress response at the hypothalamic level, then epinephrine might well exert a direct effect on the hypophysis as indicated by Long (McDermott *et al.*) and confirmed by Fortier (1951b). However, our experiments do not preclude the hypothalamus itself as the principal site of epinephrine action. If subcutaneous epinephrine, to be effective, must stimulate the release of endogenous epinephrine from the adrenal medulla (McDermott *et al.*), our results with D-ol would favor the hypothalamus as the locus of action of extrinsic epinephrine. As mentioned earlier the findings of Hume in the dog, DeGroot in the mouse, and especially, those of Porter in the cat, point to the hypothalamus as the essential target on which epinephrine exerts its hypophysis-stimulating action. The question of whether this is true also in the rat or whether species differences account for the discrepancies, demands further work.

It is interesting that the mechanisms for stimulating release of ACTH and LH both include adrenergic mediators. The former appears to have a lower threshold and to utilize systemic epinephrine from the adrenal medulla, whereas LH release seems to require a higher level of adrenergic mediator perhaps produced locally in the hypothalamo-hypophyseal region (Markee *et al.*, 1948; Sawyer, Markee and Townsend, 1949). One might expect from these conditions that a stimulus strong enough to induce LH release would "fire" the ACTH mechanism, and such appears to be the case. Harris (1952) reports that the mating stimulus in the rabbit stimulates ACTH release as well as ovulation.

Our results are consistent with the sequence of autonomic and metabolic phases of Long's dual hypothesis. The autonomic phase appears to depend on circulating epinephrine and is blocked by agents which interfere with endogenous secretion of epinephrine in response to non-specific stress. An adrenergic blocking agent may also prevent the autonomic phase in response to extrinsic epinephrine administration. Neither type of blocking agent, however, eliminates the metabolic phase of ACTH secretion.

SUMMARY

A study was made of the effects of two Dibenamine analogues, SKF-501 (N-[9-Fluorenyl]-N-ethyl-β-chlorethylamine hydrochloride) and 2-dibenzylaminoethanol (D-ol) on the eosinophil responses to epinephrine, formalin, and cold stress in rats. SKF-501 is a potent adrenergic blocking agent but D-ol, while affecting the central nervous system, lacks adrenolytic activity. Both agents act themselves as alarming stimuli, inducing marked eosinopenia at 4 hours. An attempt to block this stress with Nembutal anesthesia failed, for Nembutal, too, induced eosinopenia.

By 24 hours after SKF-501 or D-ol injection, the eosinophil count is back to normal but the adrenergic blocking capacity of SKF-501 is still high. At this time the usual effects of formalin and cold stress on ACTH release, as reflected in the 4 hour eosinophil response, are partially blocked by either SKF-501 or D-ol. The former, but not D-ol, also blocks the effects of injected epinephrine. Both blocking agents prevent the 1-hour response to formalin, but again only SKF-501 eliminates the 1-hour response to epinephrine.

The effectiveness of D-ol is considered to depend on its capacity to prevent the secretion of endogenous epinephrine by central blockade of the stressful stimulus. SKF-501 may share this capacity but it has in addition the ability to block the effects of epinephrine on its target organs. The results are consistent with the theory that endogenous epinephrine stimulates an autonomic phase of pituitary ACTH release, to be followed by a metabolic phase relatively independent of epinephrine secretion.

Acknowledgements

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NOTES AND COMMENTS

ADSORPTION OF OXYTOCIN UPON OXIDIZED CELLULOSE

In the course of using oxidized cellulose for selective adsorption and elution of adrenocorticotrophic hormone, by the method of Astwood *et al.* (1951), it was noted that the oxytocic contaminant of the ACTH was removed along with adrenocorticotrophic activity, but unlike the corticotrophic factor it was not appreciably eluted with 0.1 N HCl (1952). Dumm (1952) has shown that the antidiuretic factor can be quantitatively adsorbed upon oxycellulose and completely eluted therefrom by methods similar to that utilized for ACTH (1).

The present communication deals with the attempts to selectively adsorb and elute the oxytocic principle of the posterior pituitary gland, using oxidized cellulose as the adsorbent.

METHODS AND RESULTS

In the initial experiment 100 mg. of lyophilized posterior pituitary gland powder was extracted with 100 ml. of 0.25% acetic acid by boiling for 1 minute and filtered. To the extract was added 2 gm. oxidized cellulose (Tennessee-Eastman, 10–12% Carboxyl) previously washed with 1 N HCl, then H_2O and finally 0.1 N HAC. The pH of the mixture was 3.3. The mixture was stirred at room temperature and aliquots removed at various times for determination of oxytocin content by the rooster blood pressure drop method (1939). A similar solution kept at room temperature with stirring for 24 hours but without oxycellulose served as a control and showed no loss in activity due to oxidative changes which may have occurred during the stirring. Results are summarized in Table 1.

TABLE 1

Duration of stirring with oxycellulose	Oxytocin Present (U.S.P. units)	Per Cent Oxytocin Removed from Solution
0	150	0
1 hour	80	53
2 hours	38	75
4 hours	42	72
24 hours	39	75

Attempts to elute the oxytocic factor from the oxycellulose with 0.1 N HCl and 1 N HCl resulted in a recovery of only 30% of the activity.

The optimum amount of oxycellulose necessary to obtain maximum adsorption was determined by stirring 100 ml. posterior pituitary extract containing 100 U.S.P. units of oxytocic activity with varying quantities of oxidized cellulose for a period of 5 hours at pH 3.3. Table 2 presents the data obtained.

TABLE 2

Amount Oxycellulose	Per Cent Adsorption
50 mg	34
200 mg	42
500 mg	55
2000 mg	72

The large amounts of oxycellulose necessary to obtain the maximum adsorption of oxytocin when extracts of posterior pituitary gland were used, were somewhat unexpected in view of the small quantities necessary to secure a similar amount of adsorption of the oxytocic contaminant from ACTH solution. Table 3 indicates this difference. The amount of oxycellulose employed in each case was 200 mg.

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TABLE 3

Fraction	Oxytocic content before adsorption U.S.P. units	Oxytocic content after adsorption U.S.P. units	Per Cent Adsorption
Crude ACTH	4200	1100	74
Crude ACTH	4200	1000	76
Crude ACTH	675	160	76

DISCUSSION

These data indicate that a maximum adsorption of about 76% of the oxytocic factor can be obtained by the use of oxycellulose. However it is to be noted that much smaller amounts of oxycellulose sufficed to remove the oxytocic factor from solutions of ACTH contaminated with oxytocin than from solutions prepared by extraction of the posterior lobe of the pituitary gland.

From the data presented it is not possible to explain the inability to entirely elute the oxytocic factor from oxycellulose using 0.1 N HCl and 1 N HCl, which observation contrasts with those of Dumm who found that the antidiuretic factor was completely eluted with 0.1 N HCl. Experiments are now in progress to determine whether buffered salt solutions may accomplish recovery of the oxytocin from the oxycellulose column. The possibility of destruction of the oxytocin during the stirring with oxycellulose by surface denaturation cannot be ignored, although this possibility is remote in view of Dumm's (1952) findings that the antidiuretic factor can be quantitatively adsorbed onto and eluted from oxycellulose columns.

It is also difficult to explain the observation that only about 75% of the oxytocin is adsorbed onto the oxycellulose even when very large amounts of oxycellulose are used.

The results obtained were utilized to accomplish removal of the major portion of oxytocic contamination from the adrenocorticotropic factor isolated from whole hog pituitary glands, and seems to offer a rather effective method for separation of the bulk of oxytocic contaminant from the adrenocorticotrophic hormone.

Alginic acid (Kelacid*) was found to have adsorptive properties similar to oxycellulose in its power to adsorb oxytocin and ACTH.

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* Kelco Co., New York City.

HIGH PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE

Among the many compounds tested for progestational activity, few have exhibited greater biological potency than progesterone (Ehrenstein, 1948).

Meystre *et al.* (1948) have shown 11-dehydroprogesterone to possess a threefold greater potency than progesterone in the Clauberg test. 17-methylprogesterone A has twice the activity of progesterone using the Corner-Allen assay method (Heuser *et al.*, 1950). Ehrenstein (1944) described the degradative synthesis of 19-norprogesterone from strophanthidin. This material was a resinous substance believed to be a mixture of stereoisomers. In a study of its physiological activity, using the Corner-Allen technique, it was reported to be "fully as active as progesterone, perhaps even more so" (Allen and Ehrenstein, 1944). The synthesis of 19-norprogesterone as the pure crystalline compound by Miramontes *et al.* (1951) made possible the present investigation of its biological activity in the rabbit.[1]

MATERIALS AND METHODS

Rabbit Bioassays:

(a) *Clauberg Test* (Clauberg, 1930). Female New Zealand white rabbits, weighing 600–800 gm., were injected subcutaneously daily with 10 mcgm. alpha estradiol in 0.5 ml. corn oil for 6 days. Following this, the progestagen was injected daily subcutaneously in corn oil solution for 5 days. Twenty-four hours after the last injection the animals were killed. Uteri were weighed and fixed in 4% formalin, sectioned at 8 microns and stained with hematoxylin and eosin.

(b) *Corner-Allen Test* (Corner and Allen, 1929). Sexually mature female New Zealand white rabbits, isolated for 4 weeks were injected subcutaneously with the test compound for 5 days following bilateral ovariectomy and biopsy of the right uterine horn. Approximately 24 hours after the last injection, the animals were killed and the uteri were treated as in (a). The uterine biopsies were taken for histological examination and only those animals were used in which the uterine biopsy showed an estrogen-stimulated endometrium. In each bioassay method, progestational proliferation was rated according to the standard scale of McPhail (1934).

RESULTS AND DISCUSSION

The activity of 19-norprogesterone was compared with that of progesterone in a series of experiments, using the Clauberg method. Table 1 presents data from two of the series.

TABLE 1. PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE USING THE CLAUBERG TEST

Series	No. of rabbits	Total dose (mcgm.)*	Progestational proliferation	
			Progesterone	19-norprogesterone
I	3	250	+3, +4, +4	—
	3	500	+4, +4, +3	—
	3	62.5	—	+3, +3, +4
	3	125.0	—	+4, +4, +4
II	3	125	0, 0, 0	—
	3	250	+3, +3, +3	—
	2	31.25	—	+3, +2
	3	62.5	—	+4, +4, +4

* Administered in 1.0 ml. corn oil solution daily over 5 days.

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[1] We are indebted to Chemical Specialties Co. Inc., and Syntex, S.A., for the steroid compounds used in these investigations.

It is apparent that a total dose of 125 mcgm. progesterone is consistently negative, whereas a comparable level of 19-norprogesterone evokes maximal progestational proliferation. A total dose of 62.5 mcgm. 19-norprogesterone produces a response similar to that of 250 mcgm. progesterone. This would indicate an activity at least four times greater than that of progesterone.

Further experiments were performed utilizing the Clauberg assay, except that the test compound was administered by stomach tube. Total oral doses of 5 mg. 19-norprogesterone were completely inactive. As had been demonstrated previously, progesterone was also ineffective at this level when given orally.

Table 2 summarizes data obtained when the Corner-Allen assay method was used. In this series, it can be seen that 500 mcgm. of progesterone produced endometrial proliferation comparable to 62.5 mcgm. 19-norprogesterone. When the latter is administered at a dose of 125 mcgm. a full response was elicited in three of five animals.

TABLE 2. PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE USING THE CORNER-ALLEN TEST

No. of rabbits	Total dose (mcgm.)*	Progestational proliferation	
		Progesterone	19-norprogesterone
8	250.0	0, 0, 0, 0, 0, 0, 0, +1	—
7	500.0	+3, +1, +1, +1, +2, +2, +1	—
5	62.5	—	+2, +0, +3, +2, +4
5	125.0	—	+3, +4, +4, +4, +1

* Administered in 1.0 ml. corn oil solution daily over 5 days.

These results indicate that the parenteral activity of 19-norprogesterone is from 4 to 8 times that of progesterone. Usually, a change in the chemical configuration of progesterone has resulted in a diminution or a complete loss of potency. In 19-norprogesterone however, the replacement of the angular methyl group with hydrogen at carbon 10 results in a decided increase in physiological activity. In some respects an analogous situation exists with noradrenalin (arterenol). Euler (1946) demonstrated that the latter is more active than adrenalin in its vasopressor effect in cats. In each instance, the substitution of hydrogen for a methyl group has enhanced the biological potency. This increased activity following demethylation has led to further investigation of nor-steroid activity (to be reported separately).

In addition to its interest as a possible factor in hormonal metabolism, 19-norprogesterone, because of its high activity, has potential clinical value.

SUMMARY

Bioassay of 19-norprogesterone employing both the Clauberg and Corner-Allen methods indicates a physiological activity 4 to 8 times greater than that of progesterone. Thus, increased potency results when the methyl group at C-10 is replaced by hydrogen.

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Public Health Service
Federal Security Agency
Bethesda, Maryland

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FAILURE OF CORTISONE TO AFFECT THE SURVIVAL OF NORMAL RATS GIVEN HISTAMINE BY CONTINUOUS INTRAVENOUS INJECTION

Our interest in the possible effect of cortisone upon the resistance of the normal rat to histamine is based upon two considerations. First, cortisone can suppress inflammation which occurs following the injury of cells or as a part of the hypersensitivity reaction. Second, it is considered that the release of histamine or of histamine-like substances from the injured cell or during the hypersensitivity reaction plays a primary role in causing inflammation (see Ingle and Baker, 1953, for review).

Although most studies of the effect of the adrenal cortical hormones upon the resistance of normal animals to histamine have yielded negative results it was considered worthwhile to extend such studies by procedures not previously used. The results of this study were negative.

Male rats of the Sprague-Dawley strain were closely paired on the basis of weight. Each rat was given a solution containing histamine hydrochloride, 0.9% sodium chloride and 3% ethanol. One rat of each pair received cortisone in the above solution (free alcohol). The solution was administered into the jugular vein by a constant injection machine at the rate of 20 ml. per 24 hours per rat. Six rats were run simultaneously. The rats were

TABLE 1. SURVIVAL TIMES OF NORMAL RATS GIVEN HISTAMINE BY CONTINUOUS INTRAVENOUS INJECTION WITH AND WITHOUT CORTISONE

Treatment		Number rats surviving (12 per group) in relation to time of infusion									
Histamine HCl, gms./24 hrs./rat	Cortisone, mgs./24 hrs./rat	Hours									
		0	6	12	18	24	30	36	42	48	54
1.0	4	12	12	12	12	12	11	9	4	3	0
1.0	0	12	12	12	12	12	10	8	5	1	0
1.5	4	12	12	12	11	8	6	4	2	0	
1.5	0	12	12	12	10	7	4	2	2	0	
2.0	4	12	12	12	8	3	0				
2.0	0	12	12	12	9	3	1	0			

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subjected to ether anesthesia during the placing of the needle into the jugular vein but were thereafter kept in activity-restriction cages without anesthesia until death. The dose of cortisone (4 mg. per rat per day) was selected from other experiments (Ingle, Nezamis and Morley, 1951) in which this dose was found to be optimal for sustaining the resistance of the adrenalectomized rat to the severe stress of muscle work.

The data are summarized in Table 1. Cortisone did not significantly affect the resistance of the rat to histamine under these conditions although it is known to suppress the inflammatory response to injury in the normal rat. The amount of histamine required to cause death in the rat under these conditions is remarkable.

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RELATIVE INEFFECTIVENESS OF ELECTRICAL STIMULATION OF THE CERVIX FOR INDUCING PSEUDOPREGNANCIES IN THE MOUSE

The occurrence of pseudopregnancy in the female rat after mating with a vasectomized male was reported by Long and Evans (1922). They also induced this condition by mechanical stimulation of the cervix with glass rods. Parkes (1926) described similar responses in the mouse. Electrical stimulation of the cervix of the rat, by means of small electrodes and a simple induction coil as reported by Shelesnyak (1931) produced a larger percentage of pseudopregnancy than mechanical irritation. This is a report of the relative ineffectiveness of electrical stimulation in producing pseudopregnancy in the mouse.

In a series of experiments requiring pseudopregnant C_3H strain mice, electrical stimulation of the cervix was used. Thirty-six C_3H mice, weighing between 24 and 26 grams, with regular 4–5 day oestrous cycles were selected at the proestrus stage. Intra-vaginal electrical stimulation with Faradic current of approximately 75 volts, peak (Phipps and Bird inductorium, A70-720) was applied blindly to the cervical area for 10 to 15 seconds. No attempt was made to visualize the os cervix for precise placing of the electrodes since in the rat this has not been necessary. Only 8 of the 36 mice became pseudopregnant (averaging 11 days duration) and in 26 of the failures which were stimulated a second time at the next proestrum only 4. Thus out of 62 attempts there were 50 failures. A second group of 40 C_3H mice was treated, using an electronic stimulator, which generated spike waves similar to the inductorium, 105 volts 50 cycles per second. Only 10 of the 40 became pseudopregnant. In the combined series of C_3H mice, 76 females where stimulated at proestrous or early oestrous and after 102 attempts, 22 pseudopregnancies were produced.

To determine whether this low percentage of pseudopregnancies following electrical stimulation of the cervix was a strain or a species difference, the same procedure was carried out on 58 Swiss mice (of which 16 were stimulated on two or more successive cycles). In 22 instances, the stimulus was through the use of the induction coil; in 52, with the electronic stimulator. In the combined group of 58 Swiss mice in which 75

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stimulations were made the number of pseudopregnancies which resulted was 19; and thus the response was similar to that of the C_3H strain.

It appears that a species difference exists between the mouse and the rat in respect to the response of the female to electrical stimulation of the cervical areas during proestrun. In the rat over 90% become pseudopregnant (Shelesnyak, 1931), while in the mouse less than 26% do.

Acknowledgement

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THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.

THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

THE LAURENTIAN HORMONE CONFERENCE

The Laurentian Hormone Conference of the AAAS will hold its 1953 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 6–11. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Mass., for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than *May 15* in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. *Nervous system-hormone interrelationships*

The central nervous system and stress-induced eosinopenia.
Dr. R. W. Porter, Univ. of Calif. School of Medicine at Los Angeles

Studies of brain metabolism and electrical activity in relation to adrenocortical physiology.
Dr. Hudson Hoagland, The Worcester Foundation for Experimental Biology

Effects of hormones on cerebral function.
Dr. D. M. Woodbury, Univ. of Utah School of Medicine

II. *Thyroid Hormone physiology and biochemistry*

Triiodothyronine in relation to thyroid physiology.
Drs. J. Gross and R. Pitt-Rivers, National Inst. for Medical Research, London, and State Univ. of New York Medical Center

Metabolic effects of thyroid hormones *in vitro*.
Drs. H. A. Lardy and G. Feldott, Univ. of Wisconsin School of Medicine

III. *Comparative endocrinology*

Endocrine mechanisms in the life of insects.
Dr. D. Bodenstein, U. S. Army Chemical Center, Maryland

Hormones produced by neurosecretory cells.
Drs. Ernst and Berta Scharrer, Univ. of Colorado Medical Center

IV. *Protein Hormones*

The preparation and chemistry of crystalline insulin.
Dr. R. G. Romans, Connaught Medical Research Laboratory

The chemistry of ACTH.
Drs. E. E. Hays and W. F. White, The Armour Research Laboratories

V. *The role of hormones in blood and blood-forming organs*

Endocrine factors and radiation-induced lymphoid tumors of mice.
Dr. Henry S. Kaplan, Stanford Univ. School of Medicine

Endocrine influences upon the formed elements of blood and blood-forming organs.
Dr. Albert S. Gordon, Washington Square College, New York Univ.

VI. *Aspects of Clinical Endocrinology*

Some problems related to ovarian function and to pregnancy.
Dr. B. Zondek, Rothschild Hadassah Univ. Hospital, Jerusalem

Clinical studies on electrolyte and fluid metabolism.
Dr. Rolf Luft, Serafimerlasarettet, Stockholm

Endocrine-metabolic studies in man.
Dr. J. W. Conn, Univ. of Michigan School of Medicine